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Doctoral dissertation

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Indigenous bacterial populations in soil suppressiveness to *Fusarium graminearum*

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Autohtone bakterijske populacije u supresivnosti zemljišta prema *Fusarium graminearum*

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Populations bactériennes indigènes dans la résistance du sol contre *Fusarium graminearum*

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INDIGENOUS BACTERIAL POPULATIONS IN SOIL SUPPRESSIVENESS TO *FUSARIUM GRAMINEARUM*

EXTENSIVE ABSTRACT

Background

Crop plants are exposed to a wide range of soil-borne phytopathogens, particularly oomycetes and fungi, which are difficult to control. Species from the fungal genus *Fusarium* are typical soil microorganisms which are among the most destructive phytopathogens. They produce a wide variety of mycotoxins, which may be present in feed and food products. The mycotoxicogenic pathogen *Fusarium graminearum* is causing significant economic losses in wheat crops throughout the world, with limited efficient control methods available.

However, certain soil microorganisms may successfully inhibit phytopathogens, thus impeding their development and consequently reducing subsequent plant infection, all of which leads to defining soils that are suppressive to disease. Although abiotic factors, such as soil physicochemical properties, may contribute to the suppression of a given pathogen, suppressiveness is essentially a phenomenon mediated by soil microorganisms, since sterilization processes turn suppressive soils into non-suppressive. Additionally, agronomic practices that increase microbial activity, such as the use of organic amendments, may enhance suppressiveness, while the use of pesticides in agricultural production diminishes the soil's ability to control diseases. In suppressive soils, disease suppression occurs despite the presence of the host plant, phytopathogen and environmental conditions favorable for disease development. Two types of soil suppressiveness have been described: general (involving the entire soil microbiota that restrict pathogen(s) growth or development, and in the case of affected fungal propagules, referred to as fungistasis) and specific (involving one or several specific microbial populations that restrict pathogen-caused disease), contrary to non-suppressive (conducive) soils, where disease regularly develops.

Suppressive soils represent a reservoir of promising biocontrol agents which could provide effective plant protection against various soil-borne phytopathogens. This potential is of great importance when considering phytopathogens like *F. graminearum*, that are causing increasing damage to crops in the on-going climate change context. It is known that soils suppressive to *Fusarium* diseases affecting various crops exist worldwide, and that there are biocontrol agents isolated from such suppressive soils. Furthermore, representatives of a range of bacterial groups carry out functions that lead to the suppression of *Fusarium*-caused diseases. For example, species from the genera *Bacillus*, *Paenibacillus* and *Streptomyces* are well known to play a role in the suppression of *Fusarium*–caused diseases through various biocontrol mechanisms (i.e., antagonism, competition, parasitism and induction of systemic resistance in plant). In addition, these bacteria also exhibit a number of plant-growth promoting properties, such as phosphorus solubilization, indole-3-acetic acid (IAA) production or 1-aminocyclopropane-1-carboxylate (ACC) deaminase production, therefore facilitating plant growth. Apart from the aforementioned species, it is known that species from the genus *Pseudomonas* have a wide range of phytobeneficial functions and play an important role in the rhizosphere. For example, various *Pseudomonas* species possess the ability to induce systemic resistance in plants, compete with pathogens through the production of siderophores and produce a large panel of antifungal substances, such as pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, phenazine, 2-hexyl-5-propyl-alkylresorcinol or hydrogen cyanide (HCN), that could inactivate or inhibit *Fusarium* growth. Moreover, *Pseudomonas* species may modulate plant growth through phytohormones production, and alter the bioavailability of nutrients, by producing ACC deaminase, solubilizing phosphates, fixing nitrogen and denitrifying. To reveal these different modes of action of phytobeneficial bacteria, genome analysis is useful because not only does it allow characterization of these

beneficial functional traits, but also bacterial identification. However, beyond these specific bacterial groups that affect pathogen and disease development, it has been shown that the higher functional and genetic diversity of the whole microbial community in soil positively contributes to soil suppressiveness.

Efficient management of plant diseases caused by *Fusarium* species is crucial in attempts to avoid crop losses and reduce mycotoxin production in food products. Research on suppressive soils, coupled with data about the agronomic practices applied, provides useful information on how to maintain or achieve greater level of suppressiveness in already suppressive soils, or how to establish suppressive character of soils at other sites. The prevalence of soil-borne pathogens in cereal crops is difficult to control due to their persistence in soil and the inefficiency of chemical treatments, therefore biological control becomes a very promising alternative for disease prevention. Insight into dynamics of soils suppressive to *Fusarium* diseases, combined with the understanding of microbial modes of action, are needed in order to develop safe, effective, and stable tools for disease management.

Objectives

Given the importance of suppressive soils (that have not yet been identified in Serbia) and the emerging pathogen *F. graminearum*, the general objective of this project was to gain a better understanding of fungistasis and suppressiveness phenomena, and to assess usefulness of suppressive and fungistatic soils as sources of bacteria with biocontrol potential.

In this context, the first objective of this research was to identify soils that are fungistatic and suppressive to *F. graminearum* in Serbia, investigate the relationship between manure amendments and the occurrence of fungistasis/suppressiveness, and compare chosen fungistatic and suppressive soils based on their fungal and prokaryotic rhizosphere diversity.

The second objective was to assess the potential of soils fungistatic to *F. graminearum* as sources of biocontrol agents. This involved isolation of bacteria of contrasted taxonomy, their characterization based on genomic and functional traits, and assessment of their wheat phytoprotective capacity against *F. graminearum*.

The third objective of this work was to identify the genomic and functional particularities of *Pseudomonas* bacteria in suppressive vs. non-suppressive soils. This was motivated by the fact that *Pseudomonas* may contribute to plant protection against *Fusarium* diseases and play a role in soil suppressiveness to these diseases, although biocontrol *Pseudomonas* have also been documented in non-suppressive soils.

Methods

To achieve the first objective, 26 fields were sampled from five locations in northern and western/central Serbia (i.e., locations near Sombor (SO), Novi Karlovci (NK), Valjevo (VA), Mionica (MI) and Čačak (CA)), aiming to have pairs of soils with contrasting manure application histories for each location. Each soil sample was divided into two parts, one that was sterilized, while the other remained non-sterilized. Subsequently, both sterilized and non-sterilized soils were inoculated with *Fusarium graminearum* MDC_Fg1 (*F. graminearum* Fg1) inoculum and incubated under controlled conditions. The control group consisted of non-sterilized, non-inoculated soils. To assess the fungistatic (fungus-inhibiting) potential of these soils, after the incubation period, a quantitative PCR (qPCR) approach with *F. graminearum*-specific primers was used to quantify the amount of *F. graminearum* Fg1 DNA present in both sterilized and non-sterilized soils. Additionally, to investigate whether fungistatic soils might also exhibit suppressiveness to damping-off disease in wheat, four soils in which fungistatic potential was associated with the addition of manure amendments were selected. These soils were re-sampled, and a greenhouse suppressiveness assay was

conducted, where one half of bread wheat (*Triticum aestivum* L.) seeds was inoculated with *F. graminearum* Fg1 spore suspension, and the other half was not. After 14 days, the number of germinated seeds was recorded, and at 28 days, the number of surviving plants, shoot length (cm), dry shoot biomass (mg), and dry shoot density were measured. Finally, rhizospheres of wheat plants from the greenhouse experiment were used to perform 16S rRNA and ITS metabarcoding. This allowed comparison of soils based on prokaryotic and fungal taxonomic composition and diversity.

To accomplish the second objective, wheat plants were cultivated in selected fungistatic and non-fungistatic soils for 28 days. After harvesting the plants, representatives of various bacterial genera were isolated from the plants' rhizospheres using both general and selective plating media. A total of 244 isolates were randomly picked, purified and subjected to an initial *in vitro* confrontation assay with *F. graminearum* Fg1. This screening procedure enabled to select bacteria for genome sequencing. Following Illumina NovaSeq sequencing and assembly, genomes of the chosen bacteria were annotated, specifically searching for genes known to be involved in biocontrol and plant growth promotion. These selected bacteria were also functionally characterized through *in vitro* assays, including the assessment of HCN and lytic enzymes production, ACC deaminase activity, phytohormones production, siderophores production and phosphate solubilization. Furthermore, bacterial ability to inhibit *F. graminearum* Fg1 conidia germination was tested, as well as the bacterial ability to produce volatile organic compounds (VOCs) that inhibit Fg1 mycelial growth. Based on the results of the latter two assays and the *in vitro* confrontation assay with *F. graminearum* Fg1, a subset of isolates was tested in greenhouse conditions to assess their capacity to protect wheat from crown-rot disease caused by *F. graminearum* Fg1. Additionally, putative biosynthetic gene clusters were identified in their genomes using the antiSMASH tool and were manually curated.

To achieve the third objective, firstly, the rhizospheres of non-inoculated wheat plants grown in selected suppressive and non-suppressive soils were used. The rhizospheric DNA was extracted and metabarcoding analysis was performed, targeting the *rpoD* gene of the *Pseudomonas fluorescens* lineage, aiming to compare diversity and composition of the fluorescent pseudomonads in suppressive vs. non-suppressive soils. Then, both *F. graminearum* Fg1-inoculated and non-inoculated rhizospheres of wheat grown in suppressive and non-suppressive soils were used to isolate *Pseudomonas*. Their DNA was extracted, and these isolates were characterized based on the *rpoD* gene, or the *rrs* gene, when the *rpoD* gene amplification was unsuccessful. Out of these, 29 *Pseudomonas* were chosen from all soil conditions, comprising the combination of four different soils with and without *F. graminearum* Fg1 inoculation. The genomes of these chosen *Pseudomonas* were sequenced using Illumina NovaSeq technique. Following whole-genome sequencing and assembly, *Pseudomonas* genomes were annotated, and genes known to be involved in biocontrol and plant growth promotion were predicted. These chosen bacteria were also functionally characterized, by performing *in vitro* assays, which included production of HCN, lytic enzymes, ACC deaminase, phytohormones, siderophores and phosphate solubilization. They were also assessed for their ability to inhibit *F. graminearum* Fg1 conidia germination, as well as for their ability to produce VOCs that inhibit *F. graminearum* Fg1 mycelial growth. From this phase of research, a subset of isolates was tested in greenhouse conditions to assess their capacity to protect wheat from crown-rot disease caused by *F. graminearum* Fg1. Finally, putative biosynthetic gene clusters found in the 29 *Pseudomonas* genomes were identified using the antiSMASH and manually curated. All obtained results were analyzed with standard statistical methods, using the analysis of variance and mean comparison tests.

Results

During this research, 10 fungistatic soils were found in Serbia, seven of which had previously received manure, and their distribution was restricted to the western/central parts of the country. At locations near Mionica (soils MI2, MI3, MI4 and MI5), manure was identified as a significant factor promoting fungistasis. Soils MI2 and MI3 which had received manure, exhibited fungistasis, while soils MI4 and MI5 which were non-manured, did not show fungistasis. A similar trend was observed in the case of soils sampled near Čačak. However, the addition of manure amendments in soils near Sombor, Novi Karlovci and Valjevo was not associated with fungistasis. The four soils from Mionica were chosen for *in planta* suppressiveness assay, revealing that soils MI2, MI3 and MI5 were suppressive, while soil MI4 was non-suppressive. Fungistasis and suppressiveness assay data enabled defining of the three soil categories: (i) soils MI2 and MI3 were fungistatic and suppressive, (ii) soil MI4 was non-fungistatic and non-suppressive, while (iii) soil MI5 was non-fungistatic but suppressive. Metabarcoding data analysis of these three soil categories indicated that microbial communities in these soils were influenced by their respective field origins, while only several taxa were soil-specific.

The isolation of 244 bacteria from fungistatic and non-fungistatic soils and an *in vitro* confrontation assay with *F. graminearum* Fg1, led to the identification of 23 isolates with potential biocontrol activity against this fungal pathogen. Among these 23 isolates, 10 originated from fungistatic and 13 from non-fungistatic soils. Whole-genome sequencing revealed that, in the fungistatic soils, three strains belonged to the genus *Pseudomonas*, one to the genus *Kosakonia*, four strains to the genus *Bacillus* and two to the genus *Priestia*. In the non-fungistatic soils, seven strains belonged to the genus *Pseudomonas*, two strains to the genus *Burkholderia*, two strains to the genus *Bacillus*, one to the genus *Brevibacillus*, and one to the genus *Chryseobacterium*. Whole-genome sequencing also revealed eight novel genomospecies. Genome annotation, together with functional assays, revealed that isolates from both fungistatic and non-fungistatic soils possessed genes and functions involved in biocontrol or plant-growth promotion. The distribution of these phytobeneficial traits was largely taxa-specific. It was also observed that only VOCs produced by strains from non-fungistatic soils inhibited mycelial growth of *F. graminearum* Fg1, while exudates from isolates from both fungistatic and non-fungistatic soils had the ability to inhibit fungal conidia germination. Finally, seven strains selected based on the results of confrontation assay, ability of strains to inhibit fungal mycelial growth through the production of VOCs or the ability of bacterial exudates to inhibit fungal conidia germination, were used in a greenhouse phytoprotection plant assay, and the biosynthetic gene clusters found in their genomes were manually curated. Results indicated that only one strain, namely *Pseudomonas GS-5* IT-194MI4 (from non-fungistatic soil) enhanced wheat germination and provided protection from crown-rot disease. However, this came at the expense of shoot biomass and chlorophyll rate. All seven strains displayed BGCs coding for siderophores and antibiotics.

In the next phase, metabarcoding analysis of the soils near Mionica, targeting the *rpoD* gene of the *P. fluorescens* group, indicated that *Pseudomonas* subcommunity differed between different soils. A total of 406 putative *Pseudomonas* obtained from all eight conditions (four soils that have or have not been inoculated with *F. graminearum* Fg1) were characterized based on *rpoD* (or *rrs*) gene. *rpoD* gene characterization succeeded with 185 isolates, yielding 65 different *rpoD* sequences. Altogether 29 *Pseudomonas* from all four MI soils and conditions (inoculated or non-inoculated with *F. graminearum* Fg1) were subjected to whole-genome sequencing, thus confirming their affiliation to the *Pseudomonas* genus and revealing 16 novel genomospecies. Two of these novel genomospecies (each with two strains from different soils) were formally described and the names *P. serbica* and *P. serboccidentalis* were proposed for them. Genome annotation and functional characterization of the 29 *Pseudomonas* revealed

that their phytobeneficial genes and functions are spread evenly among strains, regardless of the experimental conditions (field of origin, inoculation with *F. graminearum* Fg1, suppressiveness status and previous manure application). *Pseudomonas* strains from all four MI soils had the ability to inhibit *F. graminearum* Fg1 mycelia development through the production of VOCs, while only strains from MI5 soil (non-fungistatic and suppressive) had the ability to inhibit fungal conidia germination. None of the *Pseudomonas* isolates conferred wheat protection from *F. graminearum* Fg1. Manual curation of BGCs found in genomes of these 29 *Pseudomonas* revealed even distribution of biosynthetic gene clusters potentially involved in biocontrol.

Conclusions

Soils fungistatic and suppressive to *F. graminearum* Fg1 disease were identified for the first time in Serbia, and manure was shown to be a significant factor promoting fungistasis in fields near Mionica, but not at other locations. Secondly, it was also demonstrated that fungistatic soils may also be suppressive, and that suppressive and non-suppressive soils shared the main prokaryotic and fungal phyla, as well as the majority of the most abundant taxa, yet several taxa were soil-specific. Thirdly, it was shown that both fungistatic and non-fungistatic soils may be a source of bacteria with antagonistic properties against *F. graminearum* and that whole-genome sequencing is a useful approach to gain insight into the biocontrol potential and taxonomic status of antagonistic strains. Fourthly, two novel *Pseudomonas* species were described, i.e., *P. serbica* and *P. serboccidentalis.* Furthermore, it was also shown that *Pseudomonas* species in both suppressive and non-suppressive soils might display similar biocontrol functions. In conclusion, the data obtained during this research may serve as a foundation for further research on soils suppressive to *F. graminearum* diseases and a basis for rhizosphere microbiome studies, resulting in a collection of thoroughly characterized bacterial strains with significant applicative potential.

Keywords: biological control, fungistasis, *Fusarium graminearum*, genomics, microbiome, PGPR, *Pseudomonas*, rhizosphere, suppressive soils

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AUTOHTONE BAKTERIJSKE POPULACIJE U SUPRESIVNOSTI ZEMLJIŠTA PREMA *FUSARIUM GRAMINEARUM*

OPŠIRNI SAŽETAK

Uvod

Poljoprivredni usevi su izloženi velikom broju zemljišnih fitopatogena, posebno oomicetama i gljivama, koje je teško kontrolisati. Vrste gljiva iz roda *Fusarium* su tipični zemljišni mikroorganizmi koji spadaju među najrazornije fitopatogene. Oni proizvode širok spektar mikotoksina, koji mogu biti prisutni u hrani i prehrambenim proizvodima. Biljni patogen *Fusarium graminearum*, koji takodje proizvodi mikotoksine, izaziva značajne ekonomske gubitke prinosa pšenice širom sveta, uz ograničene dostupne metode kontrole.

Međutim, određeni mikroorganizmi u zemljištu mogu uspešno da inhibiraju fitopatogene, ometajući njihov razvoj i posledično smanjujući pojavu infekcije biljaka, što sve dovodi do definisanja supresivnih zemljišta. Iako abiotički faktori, kao što su fizičko-hemijska svojstva zemljišta, mogu doprineti supresiji određenog patogena, supresivnost je u suštini fenomen posredovan zemljišnim mikroorganizmima, pošto procesi sterilizacije pretvaraju supresivno u nesupresivno zemljište. Takođe, agronomske prakse koje pospešuju mikrobnu aktivnost, npr. upotreba stajnjaka, mogu povećati supresivnost, dok upotreba pesticida u poljoprivrednoj proizvodnji umanjuje prirodnu sposobnost zemljišta da kontroliše bolesti. U supresivnim zemljištima, pojava suzbijanja bolesti se dešava uprkos prisustvu biljke domaćina, fitopatogena i uslova sredine pogodnih za razvoj bolesti. Opisana su dva tipa supresivnosti zemljišta: opšta (uključuje celokupan mikrobiom zemljišta koji ograničava rast ili razvoj patogena, i u slučaju patogenih gljiva se naziva fungistaza) i specifična (uključuje jednu ili više specifičnih mikrobnih populacija koje ograničavaju bolest uzrokovanu patogenom), za razliku od nesupresivnih zemljišta (tj. konducivnih), gde se bolest redovno razvija.

Supresivna zemljišta predstavljaju rezervoar obećavajućih biokontrolnih agenasa koji bi mogli da obezbede efikasnu zaštitu biljaka od različitih zemljišnih fitopatogena. Ovaj potencijal je od velike važnosti kada su u pitanju fitopatogeni kao što je *F. graminearum*, koji izazivaju sve veću štetu usevima u kontekstu rastućih klimatskih promena. Poznato je da zemljišta supresivna prema bolestima izazvanim gljivom *Fusarium* postoje širom sveta i da su iz tih zemljišta izolovani biokontrolni agensi. Štaviše, predstavnici niza bakterijskih rodova vrše funkcije koje dovode do supresije bolesti izazvanim ovom gljivom. Na primer, poznato je da vrste iz rodova *Bacillus*, *Paenibacillus* i *Streptomyces* igraju ulogu u suzbijanju bolesti izazvanim gljivom *Fusarium* kroz različite mehanizme biokontrole (tj. antagonizam, kompeticija, parazitizam i indukcija sistemske otpornosti biljke). Pored toga, ove bakterije takođe pokazuju brojna svojstva koja podstiču rast biljaka, kao što je solubilizacija fosfata, proizvodnja indol-3-sirćetne kiseline (IAA) ili proizvodnja 1-aminociklopropan-1-karboksilat (ACC) deaminaze. Pored pomenutih vrsta, poznato je da vrste iz roda *Pseudomonas* imaju širok spektar korisnih funkcija i igraju važnu ulogu u rizosferi. Na primer, različite vrste roda *Pseudomonas* imaju sposobnost da indukuju sistemsku otpornost biljke, stupaju u kompeticiju sa patogenima kroz proizvodnju siderofora i mogu proizvesti široki spektar antifungalnih supstanci, kao što su pioluteorin, pirolnitrin, 2,4-diacetilfloroglucinol, fenazin, 2 -heksil-5-propil-alkilresorcinol ili cijanovodonik (HCN), koji mogu da inaktiviraju ili inhibiraju rast *Fusarium*. Štaviše, vrste roda *Pseudomonas* mogu modulirati rast biljke putem proizvodnje fitohormona i uticati na bioraspoloživost nutrijenata, proizvodnjom ACC deaminaze, solubilizacijom fosfata, azotofiksacijom i denitrifikacijom. Kako bi se otkrili različiti načini delovanja korisnih bakterija, analiza genoma je korisna jer ne samo da omogućava karakterizaciju ovih korisnih funkcionalnih osobina, već i identifikaciju bakterija. Međutim, pored ovih specifičnih bakterijskih grupa koje deluju na razvoj patogena i bolesti, pokazalo se

da veći funkcionalni i genetički diverzitet cele mikrobne zajednice u zemljištu pozitivno doprinosi supresivnosti zemljišta.

Efikasna kontrola vrsta roda *Fusarium* je ključna u pokušaju da se izbegnu gubici prinosa i da se smanji proizvodnja mikotoksina u prehrambenim proizvodima. Istraživanja supresivnih zemljišta, zajedno sa podacima o primenjenim agronomskim praksama, pružaju korisne informacije o tome kako održati ili postići veći nivo supresivnosti u već supresivnim zemljištima, ili kako uspostaviti supresivni karakter zemljišta na drugim lokacijama. Zemljišne patogene je teško kontrolisati zbog njihove postojanosti u zemljištu i neefikasnosti hemijskih tretmana, stoga biološka kontrola postaje vrlo obećavajuća alternativa za prevenciju bolesti. Razumevanje dinamike zemljišta supresivnih prema gljivi *Fusarium*, u kombinaciji sa razumevanjem mikrobnih mehanizama delovanja, je neophodno kako bi se razvili sigurni, efikasni i stabilni alati za upravljanje bolestima.

Ciljevi

S obzirom na značaj supresivnih zemljišta (koja do sada nisu dokumentovana u Srbiji) i patogena *F. graminearum*, opšti cilj ovog istraživanja je bio bolje razumevanje fungistaze i fenomena supresivnih zemljišta, kao i procena korisnosti supresivnih i fungistatičnih zemljišta kao izvora biokontrolnih bakterija.

Stoga je prvi cilj ovog istraživanja bio da se identifikuju zemljišta koja su fungistatična i supresivna prema *F. graminearum* u Srbiji, da se ispita povezanost upotrebe stajnjaka i pojave fungistaze/supresivnosti, i da se uporedi diverzitet gljiva i prokariota u odabranim fungistatičnim i supresivnim zemljištima.

Drugi cilj je bio da se proceni potencijal zemljišta fungistatičnih prema *F. graminearum* kao izvora biokontrolnih agenasa. U tu svrhu su izolovane bakterije koje pripadaju različitim taksonomskim kategorijama, one su okarakterisane na osnovu genomskih i funkcionalnih osobina i procenjen je njihov potencijal u zaštiti pšenice od *F. graminearum*.

Treći cilj ovog rada bio je da se identifikuju genomske i funkcionalne specifičnosti bakterija roda *Pseudomonas*, poreklom iz supresivnih i nesupresivnih zemljišta. Ovo je motivisano činjenicom da *Pseudomonas* može doprineti zaštiti biljaka od bolesti izazvanim gljivom *Fusarium* i igrati ulogu u supresivnim zemljištima, iako su biokontrolni *Pseudomonas* takođe dokumentovani i u nesupresivnim zemljištima.

Metode

Kako bi se postigao prvi cilj, uzorkovano je 26 poljoprivrednih zemljišta sa pet lokacija u severnoj i zapadno/centralnoj Srbiji (tj. lokacije u blizini Sombora (SO), Novih Karlovaca (NK), Valjeva (VA), Mionice (MI) i Čačka (CA)), sa ciljem da se na svakoj lokaciji uzorkuju zemljišta na kojima je primenjivan i na kojima nije primenjivan stajnjak. Svaki uzorak je podeljen na dva dela, jedan deo je sterilisan, dok drugi nije. Nakon toga, i sterilisana i nesterilisana zemljišta su inokulisana inokulumom *Fusarium graminearum* MDC_Fg1 (*F. graminearum* Fg1) i inkubirana u kontrolisanim uslovima. Kontrolnu grupu činila su nesterilisana, neinokulisana zemljišta. Da bi se procenio fungistatični (inhibirajući) potencijal ovih zemljišta, nakon perioda inkubacije, korišćen je kvantitativni PCR sa prajmerima specifičnim za *F. graminearum*, kako bi se kvantifikovala količina *F. graminearum* Fg1 DNK prisutne u sterilisanim i nesterilisanim zemljištima. Pored toga, da bi se ispitalo da li fungistatična zemljišta mogu takođe biti i supresivna, izabrana su četiri zemljišta u kojima je fungistatični potencijal povezan sa dodavanjem stajnjaka. Ova zemljišta su ponovo uzorkovana i sproveden je test supresivnosti u kome je pšenica (*Triticum aestivum* L.) gajena nakon što je polovina semena inokulisana suspenzijom spora *F. graminearum* Fg1, a druga polovina nije. Nakon 14 dana evidentiran je broj proklijalih semena, a nakon 28 dana, evidentiran je broj preživelih

biljaka, dužina izdanaka (cm), biomasa suvih izdanaka (mg) i gustina suvih izdanaka. Konačno, rizosfere pšenice iz ovog testa supresivnosti korišćene su za 16S rRNA i ITS metabarkodiranje, što je omogućilo poređenje zemljišta na osnovu taksonomskog sastava i diverziteta prokariota i gljiva.

Kako bi se postigao drugi cilj, pšenica je gajena u odabranim fungistatičnim i nefungistatičnim zemljištima tokom 28 dana. Nakon žetve biljaka, predstavnici različitih bakterijskih rodova su izolovani iz rizosfera ovih biljaka, korišćenjem standardnih i selektivnih podloga. Nasumično odabrani izolati su prečišćeni i podvrgnuti konfrontacijskom *in vitro* testu sa *F. graminearum* Fg1. Ova procedura selekcije je omogućila odabir bakterija za sekvenciranje genoma. Nakon Illumina NovaSeq sekvenciranja i asemblinga, genomi odabranih bakterija su anotirani, tražeći gene za koje se zna da su uključeni u biokontrolu i pospešivanje rasta biljaka. Ove odabrane bakterije su takođe funkcionalno okarakterisane putem *in vitro* testova, uključujući utvrđivanje proizvodnje HCN i litičkih enzima, aktivnosti ACC deaminaze, proizvodnje fitohormona, proizvodnje siderofora i solubilizacije fosfata. Pored toga, testirana je sposobnost bakterija da inhibiraju germinaciju konidija *F. graminearum* Fg1, kao i sposobnost bakterija da proizvedu isparljiva organska jedinjenja (VOC) koja inhibiraju rast micelijuma Fg1. Na osnovu rezultata poslednja dva testa i konfrontacijskog *in vitro* testa sa *F. graminearum* Fg1, deo izolata je testiran u uslovima staklenika kako bi se procenila njihova sposobnost da zaštite pšenicu od truleži stabla izazvane *F. graminearum* Fg1. Pored toga, biosintetički genski klasteri su identifikovani u njihovim genomima pomoću antiSMASH alata i ručno su anotirani.

Kako bi se postigao treći cilj, pošlo se od rizosfere neinokulisane pšenice koja je gajena u odabranim supresivnim i nesupresivnim zemljištima. Izolovana je DNK iz rizosfere i izvršeno je metabarkodiranje koristeći *rpoD* gen grupe *Pseudomonas fluorescens*, sa ciljem da se uporedi diverzitet i sastav fluorescentnih pseudomonada u supresivnim i nesupresivnim zemljištima. Zatim su rizosfere pšenice, gajene u supresivnim i nesupresivnim zemljištima, inokulisanim i neinokulisanim fitopatogenom *F. graminearum* Fg1, korišćene za izolaciju *Pseudomonas*. Njihova DNK je izolovana, i ovi izolati su okarakterisani na osnovu *rpoD* gena, ili *rrs* gena, ukoliko amplifikacija *rpoD* gena nije bila uspešna. Od svih ovih *Pseudomonas*, 29 je odabrano iz svih eksperimentalnih uslova (četiri različita zemljišta, sa i bez inokulacije *F. graminearum* Fg1). Genomi odabranih *Pseudomonas* su sekvencirani korišćenjem Illumina NovaSeq tehnike. Nakon sekvenciranja i asemblinga, genomi *Pseudomonas* su anotirani, tražeći gene za koje se zna da su uključeni u biokontrolu i stimulaciju rasta biljaka. Ove izabrane bakterije su takođe funkcionalno okarakterisane izvođenjem *in vitro* testova, koji su uključivali proizvodnju HCN, litičkih enzima, ACC deaminaze, fitohormona, siderofora i solubilizaciju fosfata. Takođe je procenjena njihova sposobnost da inhibiraju germinaciju konidija *F. graminearum* Fg1, kao i njihova sposobnost da proizvedu VOC koji inhibiraju rast micelijuma *F. graminearum* Fg1. Iz ove faze istraživanja, deo izolata je testiran u uslovima staklenika kako bi se procenio njihov kapacitet da zaštite pšenicu od truleži stabla izazvane *F. graminearum* Fg1. Na kraju, biosintetički genski klasteri su identifikovani u genomima 29 *Pseudomonas* pomoću antiSMASH alata i ručno su anotirani. Svi dobijeni rezultati su analizirani standardnim statističkim metodama, korišćenjem analize varijanse i testova poređenja srednje vrednosti.

Rezultati

Tokom ovog istraživanja, u Srbiji je dokumentovano 10 fungistatičnih poljoprivrednih zemljišta, čija je rasprostranjenost bila ograničena na zapadne/centralne delove zemlje. Od ovih 10 fungistatičnih zemljišta, sedam je prethodno tretirano stajnjakom. Na poljoprivrednim zemljištima u blizini Mionice (zemljišta MI2, MI3, MI4 i MI5), stajnjak je identifikovan kao značajan faktor koji podstiče fungistazu. Zemljišta MI2 i MI3 u koja je unošen stajnjak su bila

fungistatična, dok zemljišta MI4 i MI5, u koja nije unošen stajnjak, nisu bila fungistatična. Sličan trend je uočen i kod poljoprivrednih zemljišta u blizini Čačka. Međutim, dodavanje stajnjaka u zemljište kod Sombora, Novih Karlovaca i Valjeva nije bilo povezano sa fungistazom. Četiri zemljišta iz Mionice su odabrana za ispitivanje supresivnosti, čime je pokazano da su zemljišta MI2, MI3 i MI5 supresivna, dok je zemljište MI4 bilo nesupresivno. Podaci dobijeni nakon testova fungistaze i supresivnosti omogućili su da se definišu tri kategorije zemljišta: (i) zemljišta MI2 i MI3 su bila fungistatična i supresivna, (ii) zemljište MI4 nije bilo fungistatično ni supresivno, dok (iii) zemljište MI5 nije bilo fungistatično, ali je bilo supresivno. Analiza podataka dobijenih metabarkodiranjem ove tri kategorije zemljišta pokazala je da mikrobne zajednice zavise od zemljišta korišćenog za izolaciju, kao i da je nekoliko taksona specifično za svaku zemljišnu kategoriju.

Izolacija 244 bakterije iz fungistatičnog i nefungistatičnog zemljišta i sprovođenje *in vitro* konfrontacijskog testa sa *F. graminearum* Fg1, omogućilo je selekciju 23 izolata sa potencijalnom biokontrolnom aktivnošću prema ovom patogenu. Među ova 23 izolata, 10 je bilo poreklom iz fungistatičnih, a 13 iz nefungistatičnih zemljišta. Sekvenciranjem celog genoma ovih izolata, otkriveno je da iz fungistatičnih zemljišta tri soja pripadaju rodu *Pseudomonas*, jedan rodu *Kosakonia*, četiri soja rodu *Bacillus* i dva rodu *Priestia*. Iz nefungistatičnog zemljišta, sedam sojeva pripada rodu *Pseudomonas*, dva rodu *Burkholderia*, dva rodu *Bacillus*, jedan rodu *Brevibacillus* i jedan rodu *Chryseobacterium*. Sekvenciranje celog genoma je takođe otkrilo osam novih vrsta. Anotacija genoma, zajedno sa funkcionalnim testovima, otkrila je da izolati iz fungistatičnog i nefungistatičnog zemljišta poseduju gene i funkcije uključene u biokontrolu i pospešivanje rasta biljaka. Distribucija ovih korisnih osobina bila je uglavnom specifična za taksonomske kategorije kojima sojevi pripadaju. Takođe je primećeno da samo VOC proizvedeni od strane sojeva iz nefungistatičnog zemljišta inhibiraju rast micelijuma *F. graminearum* Fg1, dok eksudati izolata iz fungistatičnih i iz nefungistatičnih zemljišta imaju sposobnost da inhibiraju germinaciju konidija gljive. Konačno, sedam sojeva odabranih na osnovu ovih rezultata su testirani u uslovima staklenika, a biosintetički genski klasteri koji se nalaze u njihovim genomima su ručno anotirani. Rezultati su pokazali da je samo jedan soj, *Pseudomonas GS-5* IT-194MI4 (iz nefungistatičnog zemljišta) poboljšao klijavost pšenice i pružio zaštitu od bolesti truleži stabla, ali na račun biomase izdanaka i količine hlorofila. Svih sedam sojeva je u svojim genomima imalo biosintetičke genske klastere koji kodiraju proizvodnju siderofora i antibiotika.

U sledećoj fazi istraživanja, metabarkodiranje poljoprivrednih zemljišta u blizini Mionice, ciljajući *rpoD* gen grupe *P. fluorescens*, pokazalo je da se zajednica *Pseudomonas* razlikuje među zemljištima. Ukupno 406 izolata, potencijalnih predstavnika roda *Pseudomonas*, dobijeno je na selektivnoj podlozi iz svih osam eksperimentalnih uslova (četiri zemljišta, inokulisana ili ne sa *F. graminearum* Fg1), i okarakterisano je na osnovu *rpoD* (ili *rrs*) gena. Karakterizacija *rpoD* gena je uspela sa 185 izolata, dajući 65 različitih sekvenci *rpoD*. Ukupno 29 *Pseudomonas* iz sva četiri MI zemljišta i eksperimentalnih uslova (inokulisanih ili ne sa *F. graminearum* Fg1) je podvrgnuto sekvenciranju celog genoma, čime je potvrđena njihova pripadnost rodu *Pseudomonas* i otkriveno je 16 novih vrsta. Dve od ovih novih vrsta (svaka sa po dva soja iz različitih zemljišta) su formalno opisane i predložena su imena *P. serbica* i *P. serboccidentalis*. Anotacija genoma i funkcionalna karakterizacija 29 *Pseudomonas* otkrila je da su njihovi korisni geni i funkcije ravnomerno raspoređeni među sojevima, bez obzira na eksperimentalne uslove (zemljište, inokulacija sa *F. graminearum* Fg1, supresivni status zemljišta i prethodna primena stajnjaka). Sojevi *Pseudomonas* iz sva četiri MI zemljišta su imali sposobnost da inhibiraju razvoj micelijuma *F. graminearum* Fg1 putem proizvodnje VOC, dok su samo sojevi iz zemljišta MI5 (nefungistatično i supresivno) imali sposobnost da inhibiraju germinaciju konidija gljive. Nijedan od *Pseudomonas* sojeva nije omogućio zaštitu pšenice od *F. graminearum* Fg1. Anotacija biosintetičkih genskih klastera pronađenih u

genomima ovih 29 *Pseudomonas* otkrila je ravnomernu distribuciju klastera potencijalno uključenih u biokontrolu.

Zaključci

Zemljišta koja su fungistatična i supresivna prema bolestima izazvanim *F. graminearum* Fg1 prvi put su identifikovana u Srbiji, a stajnjak se pokazao kao značajan faktor koji podstiče fungistazu na poljoprivrednim zemljištima u okolini Mionice, ali ne i na drugim lokacijama. Takođe je pokazano da fungistatična zemljišta mogu biti i supresivna, kao i da supresivna i nesupresivna zemljišta dele glavne prokariotske i fungalne filume i većinu najzastupljenijih taksona, ali da je nekoliko taksona specifično za svako zemljište. Treće, pokazalo se da i fungistatična i nefungistatična zemljišta mogu biti izvor bakterija sa antagonističkim svojstvima protiv *F. graminearum*, da je sekvenciranje celog genoma korisno za sticanje uvida u potencijalne mehanizme biokontrole, kao i za otkrivanje taksonomskog statusa ovih sojeva. Četvrto, opisane su dve nove vrste *Pseudomonas*, odnosno *P. serbica* i *P. serboccidentalis*. Takođe je pokazano da vrste roda *Pseudomonas* iz supresivnih i nesupresivnih zemljišta mogu imati sličan biokontrolni potencijal. Sve u svemu, podaci dobijeni tokom ovog istraživanja mogu poslužiti kao osnova za dalja istraživanja zemljišta koja su supresivna prema bolestima izazvanim gljivom *F. graminearum* i osnova za proučavanje mikrobioma rizosfere, rezultujući kolekcijom temeljno okarakterisanih bakterijskih sojeva sa značajnim aplikativnim potencijalom.

Ključne reči: biološka kontrola, fungistaza, *Fusarium graminearum*, genomika, mikrobiom, PGPR, *Pseudomonas*, rizosfera, supresivna zemljišta

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POPULATIONS BACTÉRIENNES INDIGÈNES DANS LA RÉSISTANCE DU SOL CONTRE *FUSARIUM GRAMINEARUM*

RÉSUMÉ SUBSTANTIEL

Introduction

Les plantes cultivées sont exposées à un multitude de phytopathogènes présents dans le sol, en particulier les oomycètes et les champignons, difficiles à contrôler. Les espèces du genre *Fusarium*, des microorganismes fongiques courants dans les sols, comptent parmi les phytopathogènes les plus destructeurs. Ils produisent une grande variété de mycotoxines, pouvant se retrouver dans les aliments pour animaux et les produits alimentaires. Le pathogène mycotoxigène *Fusarium graminearum* engendre d'importantes pertes économiques dans les cultures de blé à travers le monde, avec des méthodes de contrôle efficaces limitées.

Cependant, certains microorganismes du sol peuvent réussir à inhiber les phytopathogènes, entravant ainsi leur développement et réduisant les infections ultérieures des plantes. Cela conduit à définir des sols résistants aux maladies. Bien que des facteurs abiotiques, tels que les propriétés physicochimiques du sol, puissent contribuer à la suppression d'un agent pathogène, la résistance est essentiellement un phénomène médié par les microorganismes du sol. En effet, la stérilisation transforme les sols résistants en sols non-résistants (dits sensibles). De plus, les pratiques agricoles qui augmentent l'activité microbienne, comme l'amendements organiques, peuvent renforcer le pouvoir résistant du sol, tandis que l'utilisation de pesticides dans la production agricole diminue sa capacité à contrôler les maladies. Dans les sols résistants, la maladie est supprimée malgré la présence de la plante hôte, du phytopathogène et des conditions environnementales favorables au développement de la maladie. Deux types de résistance des sols ont été décrits : la résistance générale, impliquant l'ensemble du microbiote du sol qui limite la croissance ou le développement d'agents pathogènes (cas de la fongistase, sol dans lesquels les propagules fongiques sont affectées) et la résistance spécifique, impliquant une ou plusieurs populations microbiennes spécifiques qui limitent les maladies causées par des agents pathogènes. Cela contraste avec les sols sensibles, où les maladies se développent régulièrement.

Les sols résistants représentent un réservoir d'agents de lutte biologique prometteurs qui pourraient fournir une protection efficace aux plantes contre divers phytopathogènes présents dans le sol. Ce potentiel revêt une grande importance, surtout face à des phytopathogènes tel que *F. graminearum*, qui causent des dommages croissants aux cultures dans le contexte actuel du changement climatique. On sait que des sols résistants aux maladies causées par *Fusarium* existent dans le monde entier, affectant diverses cultures, et que des agents de lutte biologique sont isolés de ces sols. En outre, des représentants de divers groupes bactériens remplissent des fonctions qui conduisent à la résistance aux maladies causées par le *Fusarium*. Par exemple, les espèces des genres *Bacillus*, *Paenibacillus* et *Streptomyces* sont bien connues pour jouer un rôle dans la résistance contre des maladies causées par *Fusarium*, grâce à divers mécanismes de contrôle biologique tel que l'antagonisme, compétition, parasitisme et induction d'une résistance systémique chez les plantes. Ces bactéries présentent également un certain nombre de propriétés favorisant la croissance des plantes, telles que la solubilisation du phosphore, la production d'acide indole-3-acétique (AIA) ou la production de 1-aminocyclopropane-1-carboxylate (ACC) désaminase. Outre les espèces mentionnées ci-dessus, il est établi que les espèces du genre *Pseudomonas* possèdent une multitude de fonctions phytobénéfiques et jouent un rôle important dans la rhizosphère. Par exemple, diverses espèces de *Pseudomonas* sont capables d'induire une résistance systémique chez les plantes, de rivaliser avec les agents pathogènes par la production de sidérophores et de générer une variété de substances antifongiques, telles que

la pyolutéorine, la pyrrolnitrine, le 2,4-diacétylphloroglucinol, la phénazine, le 2-hexyl-5-propyle-l'alkylrésorcinol ou le cyanure d'hydrogène (HCN), pouvant inactiver ou inhiber la croissance de *Fusarium*. De plus, les espèces de *Pseudomonas* peuvent moduler la croissance des plantes en produisant des phytohormones et elles peuvent modifier la biodisponibilité des nutriments, par example en produisant de l'ACC désaminase, en solubilisant les phosphates, en fixant l'azote et/ou en dénitrifiant. Pour élucider ces divers modes d'action des bactéries bénéfiques pour les plantes, l'analyse génomique est précieuse. Elle permet non seulement de caractériser ces traits fonctionnels bénéfiques, mais également d'identifier les bactéries elles-mêmes. Cependant, au-delà de ces groupes bactériens spécifiques qui influent sur le développement des agents pathogènes et des maladies, il a été démontré que la plus grande diversité fonctionnelle et génétique de l'ensemble de la communauté microbienne du sol contribue positivement à la résistance du sol.

Une gestion efficace des maladies des plantes causées par les espèces de *Fusarium* est cruciale pour tenter de minimiser les pertes de récoltes et de réduire la production de mycotoxines dans les produits alimentaires. La recherche sur les sols résistants, associée aux données sur les pratiques agronomiques appliquées, offre des informations précieuses sur la manière de maintenir ou d'atteindre un niveau plus élevé de résistance dans des sols déjà résistants, ou sur la manière d'établir le caractère résistant des sols sur d'autres sites. La prévalence des agents pathogènes du sol dans les cultures céréalières est difficile à maîtriser en raison de leur persistance dans le sol et de l'inefficacité des traitements chimiques. La lutte biologique devient donc une alternative très prometteuse pour la prévention des maladies. Une compréhension approfondie de la dynamique des sols résistants aux maladies causées par *Fusarium*, combinée à la connaissance des modes d'action microbiens, est nécessaire pour développer des outils sûrs, efficaces et stables pour la gestion des maladies.

Objectifs

Compte tenu de l'importance des sols résistants (en Serbie, où jusqu'alors aucun sol résistant n'était identifié) et du pathogène émergent *F. graminearum*, l'objectif général de ce projet était de mieux comprendre les phénomènes de fongistase et de résistance, et d'évaluer l'utilité des sols résistants et fongistatiques comme les sources de bactéries ayant un potentiel de biocontrôle.

Dans ce contexte, le premier objectif de cette recherche était d'identifier les sols fongistatiques et résistants de *F. graminearum* en Serbie, d'étudier la relation entre les amendements du fumier et l'apparition de fongistase/résistance, et de comparer les sols fongistatiques et résistants choisis en fonction de leur diversité des rhizosphères fongiques et procaryotes.

Le deuxième objectif était d'évaluer le potentiel des sols fongistatiques envers *F. graminearum* comme sources d'agents de biocontrôle. Cela impliquait l'isolement de bactéries de taxonomies contrastées, leur caractérisation basée sur des traits génomiques et fonctionnels, et l'évaluation de leur capacité phytoprotectrice du blé contre *F. graminearum*.

Le troisième objectif de ce travail était d'identifier les particularités génomiques et fonctionnelles de la bactérie *Pseudomonas* dans des sols résistants ou sensibles. Cette démarche était motivée par le fait que *Pseudomonas* peut contribuer à la protection des plantes contre les maladies causées par *Fusarium* et jouer un rôle dans la résistance aux maladies par le sol, bien que le contrôle biologique de *Pseudomonas* ait également été documenté dans des sols sensibles.

Méthodes

Pour atteindre le premier objectif, 26 champs ont été échantillonnés dans cinq emplacements du nord et de l'ouest/centre de la Serbie (c'est-à-dire des emplacements proches de Sombor (SO), Novi Karlovci (NK), Valjevo (VA), Mionica (MI) et Čačak (CA)), visant à obtenir des paires de sols avec des historiques d'application de fumier contrastés pour chaque emplacement. Chaque échantillon de sol a été divisé en deux fractions, l'une stérilisée et l'autre non stérilisée. Par la suite, les sols stérilisés et non-stérilisés ont été inoculés avec l'inoculum de *Fusarium graminearum* MDC_Fg1 (*F. graminearum* Fg1) et incubés dans des conditions contrôlées. Le groupe témoin était constitué de sols non stérilisés et non-inoculés. Pour évaluer le potentiel fongistatique (inhibiteur de champignons) de ces sols, après la période d'incubation, une approche PCR quantitative (qPCR) avec des amorces spécifiques de *F. graminearum* a été utilisée pour quantifier la quantité d'ADN Fg1 de *F. graminearum* présente dans les sols stérilisés et sols non-stérilisés. De plus, pour déterminer si les sols fongistatiques pourraient également présenter un pouvoir résistante contre la fonte des semis chez le blé, quatre sols dans lesquels le potentiel fongistatique était associé à l'ajout d'amendements de fumier ont été sélectionnés. Ces sols ont été ré-échantillonnés et un test de résistance en serre a été effectué, où la moitié des graines de blé tendre (*Triticum aestivum* L.) a été inoculée avec une suspension de spores *F. graminearum* Fg1, et l'autre moitié ne l'a pas été. Après 14 jours, le nombre de graines germées a été enregistré et, à 28 jours, le nombre de plantes survivantes, la longueur des pousses (cm), la biomasse des pousses sèches (mg) et la densité des pousses sèches ont été mesurées. Enfin, des rhizosphères de plants de blé provenant de l'expérience en serre ont été utilisées pour effectuer le métabarcoding de l'ARNr 16S et de l'ITS. Cela a permis de comparer les sols sur la base de la composition et de la diversité taxonomiques des procaryotes et des champignons.

Pour atteindre le deuxième objectif, des plants de blé ont été cultivés dans des sols fongistatiques et non-fongistatiques sélectionnés, pendant 28 jours. Après la récolte des plantes, des représentants de divers genres bactériens ont été isolés des rhizosphères des plantes en utilisant des milieux d'étalement généraux et sélectifs. Au total, 244 isolats ont été prélevés au hasard, purifiés et soumis à un premier test de confrontation *in vitro* avec *F. graminearum* Fg1. Cette procédure de criblage a permis de sélectionner des bactéries pour le séquençage du génome. Après le séquençage et l'assemblage d'Illumina NovaSeq, les génomes des bactéries choisies ont été annotés, recherchant spécifiquement les gènes connus pour être impliqués dans le biocontrôle et la promotion de la croissance des plantes. Ces bactéries sélectionnées ont également été caractérisées fonctionnellement par des tests *in vitro*, notamment l'évaluation de la production de HCN et d'enzymes lytiques, de l'activité de l'ACC désaminase, de la production de phytohormones, de la production de sidérophores et de la solubilisation du phosphate. En outre, la capacité bactérienne à inhiber la germination des conidies Fg1 de *F. graminearum* a été testée, ainsi que la capacité bactérienne à produire des composés organiques volatils (VOC) qui inhibent la croissance mycélienne Fg1. D'après les résultats de ces deux derniers essais et de l'essai de confrontation *in vitro* avec *F. graminearum* Fg1, un sous-ensemble d'isolats a été testé en serre pour évaluer leur capacité à protéger le blé contre la pourriture du collet causée par *F. graminearum* Fg1. De plus, des groupes de gènes biosynthétiques putatifs (BGCs) ont été identifiés dans leurs génomes à l'aide de l'outil antiSMASH et ont été annotés manuellement.

Pour atteindre le troisième objectif, les rhizosphères de plants de blé non-inoculés cultivés dans des sols résistants et sensibles ont été utilisés. L'ADN rhizosphérique a été extrait et une analyse de métabarcodes a été réalisée, ciblant le gène *rpoD*, dans le but de comparer la diversité et la composition des communautés de *Pseudomonas fluorescens* dans des sols résistants et sensibles. En parallèle, des rhizosphères de blé inoculées et non-inoculées par *F. graminearum* Fg1, cultivées dans des sols résistants et sensibles ont été

utilisées pour isoler des *Pseudomonas*. Ces isolats ont été caractérisés sur la base du gène *rpoD*, ou du gène *rrs*, lorsque l'amplification du gène *rpoD* a échoué. Parmi des *Pseudomonas* isolées, 29 ont été sélectionnées en fonctions de leurs espèces et répartition dans toutes les conditions testées (combinaison de quatre sols différents avec et sans inoculation de *F. graminearum* Fg1). Les génomes des 29 *Pseudomonas* ont été séquencés à l'aide de la technique Illumina NovaSeq. Après le séquençage et l'assemblage du génome entier, les génomes de *Pseudomonas* ont été annotés et les gènes connus pour être impliqués dans le contrôle biologique et la promotion de la croissance des plantes ont été prédits. Ces bactéries choisies ont également été caractérisées fonctionnellement en effectuant des tests *in vitro* comprenant la production de HCN, d'enzymes lytiques, d'ACC désaminase, de phytohormones, de sidérophores et la solubilisation du phosphate. Ils ont également été évalués pour leur capacité à inhiber la germination des conidies *F. graminearum* Fg1, ainsi que pour leur capacité à produire des VOC qui inhibent la croissance mycélienne de *F. graminearum* Fg1. À partir de cette phase de recherche, un sous-ensemble d'isolats a été testé en serre pour évaluer leur capacité à protéger le blé contre la pourriture du collet causée par *F. graminearum* Fg1. Enfin, des groupes des BGCs trouvés dans les 29 génomes de *Pseudomonas* ont été identifiés à l'aide de l'antiSMASH et annotés manuellement. Tous les résultats obtenus ont été analysés avec des méthodes statistiques standards, en utilisant des tests d'analyse de variance et de comparaison de moyennes.

Résultats

Au cours de cette recherche, 10 sols fongistatiques ont été découverts en Serbie, dont sept avaient déjà reçu du fumier, et leur répartition était limitée aux régions occidentales et centrales du pays. Aux endroits proches de Mionica (sols MI2, MI3, MI4 et MI5), le fumier a été identifié comme un facteur important favorisant la fongistase. Les sols MI2 et MI3 qui avaient reçu du fumier était fongistatique, tandis que les sols MI4 et MI5 qui n'étaient pas fumés - n'était pas fongistatique. Une tendance similaire a été observée dans le cas des sols échantillonnés près de Čačak. Cependant, l'ajout de fumier dans les sols proches de Sombor, Novi Karlovci et Valjevo n'a pas été associé à une fongistase. Les quatre sols de Mionica ont été choisis pour un test de résistance *in planta*, révélant que les sols MI2, MI3 et MI5 étaient résistants, tandis que le sol MI4 n'était pas résistant. Les données des tests de fongistase et de résistance ont permis de définir les trois catégories de sols : (i) les sols MI2 et MI3 étaient fongistatiques et résistants, (ii) le sol MI4 était non-fongistatique et non-résistants, tandis que (iii) le sol MI5 était non-fongistatique mais résistant. L'analyse des données de métabarcodage de ces trois catégories de sols a indiqué que les communautés microbiennes de ces sols étaient influencées par leurs origines respectives sur le terrain, alors que seuls quelques taxons étaient spécifiques au sol.

L'isolement de 244 bactéries provenant de sols fongistatiques et non-fongistatiques et un test de confrontation *in vitro* avec *F. graminearum* Fg1 ont conduit à l'identification de 23 isolats présentant une activité potentielle de biocontrôle contre ce champignon pathogène. Parmi ces 23 isolats, 10 provenaient de sols fongistatiques et 13 de sols non-fongistatiques. Le séquençage du génome entier a révélé que, dans les sols fongistatiques, trois souches appartenaient au genre *Pseudomonas*, une au genre *Kosakonia*, quatre souches au genre *Bacillus* et deux au genre *Priestia*. Dans les sols non-fongistatiques, sept souches appartenaient au genre *Pseudomonas*, deux souches au genre *Burkholderia*, deux souches au genre *Bacillus*, une au genre *Brevibacillus* et une au genre *Chryseobacterium*. Le séquençage du génome entier a également révélé huit nouvelles espèces génomiques. L'annotation du génome, ainsi que les tests fonctionnels, ont révélé que les isolats provenant de sols fongistatiques et non-fongistatiques possédaient des gènes et des fonctions impliqués dans le contrôle biologique ou la promotion de la croissance des plantes. La distribution de ces

caractères phytobénéfiques était en grande partie spécifique aux taxons. Il a également été observé que seuls les VOC produits par des souches provenant de sols non fongistatiques inhibaient la croissance mycélienne de *F. graminearum* Fg1, tandis que les exsudats d'isolats provenant de sols fongistatiques et non-fongistatiques avaient la capacité d'inhiber la germination des conidies fongiques. Enfin, sept souches sélectionnées sur la base des résultats d'un essai de confrontation, de leur capacité à inhiber la croissance mycélienne fongique par la production de VOC ou de la capacité des exsudats bactériens à inhiber la germination des conidies fongiques, ont été utilisées dans un essai de phytoprotection des plantes en serre, et des BGCs trouvés dans leurs génomes ont été annotés manuellement. Les résultats ont indiqué qu'une seule souche, *Pseudomonas* GS-5 IT-194MI4 (provenant d'un sol non-fongistatique), a amélioré la germination du blé et a assuré une protection contre la pourriture du collet. Cependant, cela s'est fait au détriment de la biomasse des pousses et du taux de chlorophylle. Les sept souches présentaient des BGC codant pour des sidérophores et des antibiotiques.

Dans la phase suivante, l'analyse des métabarcodes des sols près de Mionica, ciblant le gène *rpoD* du groupe *P. fluorescens*, a indiqué que la sous-communauté de *Pseudomonas* différait entre les différents sols. Au total, 406 *Pseudomonas* putatifs obtenus dans les huit conditions (quatre sols ayant ou non été inoculés avec *F. graminearum* Fg1) ont été caractérisés sur la base du gène *rpoD* (ou *rrs*). La caractérisation du gène *rpoD* a réussi avec 185 isolats, donnant 65 séquences *rpoD* différentes. Au total, 29 *Pseudomonas* provenant des quatre sols et conditions MI (inoculés ou non avec *F. graminearum* Fg1) ont été soumis au séquençage du génome entier, confirmant ainsi leur affiliation au genre *Pseudomonas* et révélant 16 nouvelles espèces génomiques. Deux de ces nouvelles génomoespèces (chacune avec deux souches provenant de sols différents) ont été formellement décrites et les noms *P. serbica* et *P. serboccidentalis* ont été proposés pour elles. L'annotation du génome et la caractérisation fonctionnelle des 29 *Pseudomonas* ont révélé que leurs gènes et fonctions phytobénéfiques sont répartis uniformément entre les souches, quelles que soient les conditions expérimentales (champ d'origine, inoculation avec *F. graminearum* Fg1, statut résistants et application antérieure de fumier). Les souches de *Pseudomonas* des quatre sols MI avaient la capacité d'inhiber le développement des mycéliums Fg1 de *F. graminearum* grâce à la production de VOC, tandis que seules les souches du sol MI5 (non-fongistatiques et résistants) avaient la capacité d'inhiber la germination des conidies fongiques. Aucun des isolats de *Pseudomonas* ne confère au blé une protection contre *F. graminearum* Fg1. Des BGC trouvés dans les génomes de ces 29 *Pseudomonas* a révélé une répartition uniforme des groupes de gènes biosynthétiques potentiellement impliqués dans le biocontrôle.

Conclusions

Premièrement, des sols fongistatiques et résistants aux maladies causées par *F. graminearum* Fg1 ont été identifiés pour la première fois en Serbie, et le fumier s'est révélé être un facteur important favorisant la fongistase dans les champs proches de Mionica, mais pas ailleurs. Deuxièmement, il a également été démontré que les sols fongistatiques pouvaient être résistants, et que les sols résistants et sensibles partageaient les principaux phylums procaryotes et fongiques, ainsi que la majorité des taxons les plus abondants, mais que plusieurs taxons étaient spécifiques à chaque type de sol. Troisièmement, il a été démontré que les sols fongistatiques et non-fongistatiques peuvent être une source de bactéries ayant des propriétés antagonistes contre *F. graminearum*, et que le séquençage du génome entier est une approche utile pour mieux comprendre le potentiel de biocontrôle et le statut taxonomique des souches antagonistes. Quatrièmement, deux nouvelles espèces de *Pseudomonas* ont été décrites, à savoir *P. serbica* et *P. serboccidentalis*. En outre, il a également été démontré que les espèces de *Pseudomonas* présentes dans des sols résistants et non-

résistants pourraient présenter des fonctions de biocontrôle similaires. Pour conclure, les données obtenues au cours de cette recherche peuvent servir de base à des recherches plus approfondies sur les sols résistants aux maladies à *F. graminearum* et à des études sur le microbiome de la rhizosphère, conduisant à une collection de souches bactériennes soigneusement caractérisées avec un potentiel applicatif important.

Mots clés: lutte biologique, fongistase, *Fusarium graminearum*, génomique, microbiome, PGPR, *Pseudomonas*, rhizosphère, sols résistants

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1. INTRODUCTION

In 1987, the World Commission on Environment and Development's report defined sustainable development as "development that meets the needs of the present without compromising the ability of future generations to meet their own needs" (Report of the World Commission on Environment and Development: Our Common Future, 1987). However, agricultural production has been facing significant challenges in recent decades. The use of agrochemicals, which is part of existing agricultural practices, poses a serious threat to the environment and results in soil pollution. Additionally, rapid urbanization and industrialization have led to a substantial decrease of agricultural areas. The excessive use of agrochemicals also contributes to climate change, specifically the greenhouse effect, through the emission of harmful gases and the deposition of toxic components into the soil. These issues emphasize the need to adopt alternative approaches in agriculture to ensure environmental sustainability and food security. Furthermore, these approaches aim to protect crops from phytopathogens and increase crop yields within the limited agricultural areas available. Therefore, research focused on the rhizospheres of plants, which serve as ecological niches for numerous beneficial microorganisms, may offer valuable insights into mitigating the consequences of intensive agriculture.

The rhizosphere, a narrow zone of soil surrounding plant roots, serves as a habitat for a diverse range of phytopathogens and beneficial microorganisms. These microorganisms interact and are directly influenced by the plant roots' exudates. Conventional agricultural practices struggle to effectively control soil-borne phytopathogens, often focusing solely on the plant-pathogen relationship and disregarding the complex interactions and the involvement of a diverse soil microbiome that can significantly impact infection outcomes. *Plant Growth Promoting Rhizobacteria* (PGPR), which are beneficial soil microorganisms, have shown success in inhibiting phytopathogens and protecting plants from infections. The role of plant-protecting soil microbiota in promoting plant health is particularly interesting in disease-suppressive soils. In these soils, phytopathogens, host plants, and favorable environmental conditions coexist without disease occurrence. Suppressive soils act as reservoirs of beneficial microorganisms that can provide effective plant protection against various soil-borne phytopathogens through several mechanisms. These microorganisms can (1) produce metabolites that are antagonistic to plant pathogens, (2) compete with pathogens for resources and space, (3) directly combat plant pathogens via hyperparasitism, and/or (4) induce plant resistance by stimulating the plant's defense mechanisms.

The biocontrol potential of suppressive soils is particularly significant when considering phytopathogens like mycotoxicogenic *Fusarium graminearum*, which is increasingly damaging crops in the context of ongoing climate change. While suppressive soils specifically targeting *F. graminearum* have not been documented thus far, suppressive soils against diseases caused by other *Fusarium* species in various crops have been identified. It has been shown that representatives of several bacterial groups perform functions that contribute to the suppression of *Fusarium*-caused diseases. For example, species belonging to the genera *Bacillus*, *Brevibacillus*, *Burkholderia*, *Chryseobacterium*, and *Kosakonia* are well-known for their role in suppressing diseases caused by *Fusarium* through diverse biocontrol mechanisms. Moreover, some of these bacteria may exhibit *Plant Growth Promoting* (PGP) properties. They can enhance plant mineral nutrition by promoting processes such as phosphorus solubilization and nitrogen fixation. Additionally, they can modify phytohormonal balance by directly producing different phytohormones, such as auxins, or indirectly, as with the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades ACC, a precursor of the plant hormone ethylene.

These activities ultimately facilitate plant growth. In addition to the aforementioned genera, it is well-known that fluorescent *Pseudomonas* species play a crucial role in the rhizosphere and suppressive soils. These bacteria exhibit a wide range of phytobeneficial functions that can contribute to inactivating or inhibiting the growth of *Fusarium* or promoting plant growth. Agricultural practices have been shown to have a significant impact on soil suppressiveness to *Fusarium* diseases by influencing the ecology of soil microbiota. Disease-suppressive soils serve as valuable models for understanding microbiota-based phytoprotection and developing sustainable plant protection strategies for soils lacking this property. Despite decades of study, soils suppressive to *Fusarium* diseases are still not well understood in terms of microbiota functioning, and knowledge in this area remains fragmented. Previous research, primarily based on culture-based methods, aimed to identify the key taxa involved in disease suppression, but many questions remain unanswered. However, advancements in next-generation sequencing and ecological network research are providing new tools to characterize suppressive soils. This opens up possibilities for using both culture-dependent and culture-independent methods to better understand the functioning of microbiota in soils suppressive to *Fusarium* diseases. By studying microbial modes of action and diversity in suppressive soils, research can contribute to the development of effective farming practices for managing *Fusarium* diseases in sustainable agriculture.

2. BIBLIOGRAPHICAL SYNTHESIS

2.1. Soil suppressiveness

2.1.1. Definition, types and significance of soil suppressiveness

Soil is a dynamic ecosystem, a complex mixture of inorganic and organic matter, inhabited by a large number of different microorganisms, plants and animals (Tešić and Todorović, 1988; Chandrashekara et al., 2012). It is widely accepted that soil has a vital role in crop productivity and health, as it represents fertile ground for microbial cooperation and a battlefield for dynamic interactions between soil-dwelling microorganisms and plants, and as such, soil is the foundation of sustainable agriculture (Raaijmakers et al., 2009). During their lifecycle, crop plants are exposed to a wide range of soil-borne phytopathogens, particularly oomycetes and fungi, which are difficult to control. However, certain soil microorganisms may successfully inhibit these phytopathogens, thus disturbing their development and consequently reducing the subsequent plant infection, all of which leads to defining soils that are suppressive to diseases (Weller et al., 2002). Soils that are suppressive to soil-borne diseases have been known for more than 70 years (Vasudeva and Roy, 1950) and they were originally defined by Baker and Cook (1974) as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil." In these soils, disease suppression is a result of the activity of phytobeneficial microorganisms (Schlatter et al., 2017), that interact with phytopathogens, thus affecting their survival, development or infection of the plant (Weller et al., 2002; Raaijmakers et al., 2009). Abiotic factors, such as soil physicochemical properties, i.e., clay content, cation exchange capacity and soil nutrient status (NH_4^+, NO_3^-, sodium, calcium, manganese and iron contents in the soil) may contribute to the control of a given pathogen (Kurek and Jaroszuk-Ściseł, 2003; Legrand et al., 2019; Meng et al., 2019). These abiotic factors influence suppressiveness through impacting the soil microbiota, and for example, it was shown that sterilization of soils with high vermiculite contents did not provide suppression of *Thielaviopsis basicola* black root rot disease in tobacco (Stutz et al., 1986; Ramette et al., 2006). Therefore, suppressiveness is essentially a phenomenon mediated by beneficial soil microorganisms, since sterilization processes convert suppressive into non-suppressive soils (i.e., conducive soils) (Garbeva et al., 2004; Jayaraman et al., 2021). In suppressive soils, disease suppression occurs despite the presence of the host plant, phytopathogen and environmental conditions convenient for disease development (Schlatter et al., 2017). Suppressive and conducive soils may be located at small geographic distances in a landscape, and differences in plant disease incidence between neighbouring fields that share similar climatic conditions and agronomic practices are attributed by the differences in the resident microbiota in these soils (Almario et al., 2014). Methodology for identification of suppressive soils includes the introduction of a given pathogen into various soils and observing the severity of disease symptoms on susceptible host plants. At the same pathogen inoculum densities, plants' health differs between the soils, thus showing the various levels of soil suppressiveness to the pathogen introduced (Alabouvette et al., 1982). The phenomenon of disease suppressiveness has been described for many soil-borne fungal pathogens, including *Gaeumannomyces graminis* var. *tritici* (Shipton, 1973), *T. basicola* (Stutz et al., 1986), *Rhizoctonia solani* (Mendes et al., 2011) and *Fusarium* (Vasudeva and Roy, 1950; Smith and Snyder, 1971; Rouxel and Sedra, 1989; Rasmussen et al., 2002; Ossowicki et al., 2020).

Two types of soil suppressiveness have been described: general and specific, contrary to non-suppressive soils, where disease regularly develops (Schlatter et al., 2017). General disease suppression takes place in the bulk soil and it refers to the activity of the entire soil microbiota that restricts growth or survival of multiple pathogens, thus limiting the subsequent plant infection and disease, and it cannot be transferred experimentally between the soils (Weller et al., 2002). Hence, all soils may present some level of general suppressiveness to soil-borne diseases, and this level depends on the soil type, agricultural practices and total microbial activity (Janvier et al., 2007; Raaijmakers et al., 2009). In the case when fungal propagules in soil are affected by the entire soil microbiota, the phenomenon is referred to as fungistasis. Contrarily, specific disease suppression refers to the suppression of pathogen-caused disease, it usually takes place in the rhizosphere, and it is related to the activity of one or several specific microbial populations (Weller et al., 2007; Almario et al., 2014; Mousa and Raizada, 2016). Specific suppressiveness may be conferred to conducive soils by inoculating them with 0.1% - 10% of suppressive soil (Garbeva et al., 2004; Raaijmakers et al., 2009). Specific suppressiveness is sometimes an intrinsic property of the soil and persists over years, despite varying ecological conditions. This natural/long-term suppressiveness is well documented for several pathosystems, for instance in the case of soils suppressive to tobacco black root rot disease caused by *T. basicola* (Stutz et al., 1986) and in the case of soils suppressive to *Fusarium* diseases (Sneh et al., 1984; Alabouvette, 1986). Besides natural suppressiveness, specific disease suppressiveness may be induced by monoculture farming practices leading to the built-up of a plant-protecting microbiota. This takes place following crop monoculture, typically after early disease outbreak, in the presence of the pathogen, and is documented in the case of pathogen *G. graminis* var. *tritici* (Weller et al., 2002) and *Fusarium* (Cha et al., 2016; Shen et al., 2022). Both general and specific soil suppressiveness can also be induced by microbial biofertilizer inoculants which alter the composition of indigenous microbiota (Xiong et al., 2017; Tao et al., 2020). Recent advances in technology, i.e., next-generation sequencing and ecological networks, have largely contributed to the discovery of many new taxa and to the understanding of microbial communities in soils suppressive and conducive to soil-borne diseases. Consequently, comparison of suppressive vs. conducive soils has evidenced differences in the occurrence or prevalence of multiple taxa, with species from the phyla *Pseudomonadota* (formerly *Proteobateria*), *Actinomycetota* (formerly *Actinobacteria*) and *Bacillota* (formerly *Firmicutes*) being frequent in suppressive soils (Kyselková et al., 2009; Cha et al., 2016; Latz et al., 2016).

It has been shown that agronomic practices that increase microbial diversity and/or activity, may enhance suppressiveness (Schlatter et al., 2017), while the use of pesticides in agricultural production reduces the soil's ability to control diseases (Zhang et al., 2022). Besides few cases when monoculture induced suppressiveness, agricultural management based on crop rotation with the non-host plants may result in reduced survival of soil-borne pathogen propagules and positively affect suppressiveness, moreover as the crop diversity may also promote diverse beneficial microbiota (Winter et al., 2014; Schlatter et al., 2017). Practice of crop rotation is suitable for pathogens that require alive host for their survival or for those with low capability of saprophytic survival, but it is not suitable in the case of pathogens that have survival forms or those that have a vast range of hosts (Janvier et al., 2007). Organic amendments like animal manure, crop residues and different composts are often used to improve soil health by delivering nutrients to the soil and also by stimulating beneficial microbiota (Mousa and Raizada, 2016). However, there is evidence that by increasing organic matter content, a pathogen survival could also be promoted, although this is rare (Termorshuizen et al., 2006). Use of organic fertilizers supplemented with beneficial microorganisms may also enhance suppressiveness, by

altering indigenous soil microbiota (Montalba et al., 2010; Cretoiu et al., 2013). Besides, a recent tendency is to reduce overcultivation and to apply conservational tillage practices that are more strategic and less excessive. Under conservational tillage, crops debris are left on the soil surface, possibly increasing the microbial biomass and activity, leading to antagonism and competition with soil-borne phytopathogens (Janvier et al., 2007). However, many soil-borne phytopathogens are able to overwinter in the crop residues which may present a reservoir of pathogen inoculum over a long time, often surpassing the period when there are no plants in the agrosystem (Bockus and Shroyer, 1998), so under such conditions, it is necessary to ensure favorable conditions for humification processes (Leplat et al., 2013). On the other hand, under conventional tillage, when crops debris are buried in the soil, the pathogen inoculum is displaced from its niche, leading to reduced survival (Janvier et al., 2007). Taken together, the effects of tillage practices on pathogen suppression are contrasting and highly dependent on interactions between environment, crop and the pathogen (Paulitz et al., 2010).

Efficient management of plant diseases is substantial in an attempt to avoid crop losses (Babadoost, 2018). The prevalence of soil-borne pathogens in cereal crops is difficult to control due to their persistence in soil and inefficiency of available control methods (De Coninck et al., 2015), therefore biological control becomes a very promising alternative for disease prevention. Suppressive soils represent a reservoir of promising biocontrol agents which could provide an effective plant protection against various soil-borne phytopathogens (Gómez Expósito et al., 2017). Research on suppressive soils, together with the data about the agronomic practices applied, provide useful information on how to maintain or achieve greater level of suppressiveness in already suppressive soils, or how to establish suppressive character of soils at other sites. Insight into dynamics of suppressive soils, together with the understanding of microbial modes of action, is needed in order to develop safe, effective, and stable tools for disease management (Gómez Expósito et al., 2017).

2.1.2. The role of rhizosphere bacteria in soil suppressiveness

Soil represents the richest known reservoir of microbial biodiversity (Curtis et al., 2002; Wang et al., 2016) and displays two compartments, i.e., the bulk soil containing microorganisms that are not affected by the roots, and the rhizosphere where soil microorganisms are under the influence of roots (and roots' rhizodeposits). Besides, the root compartments are also inhabited by microorganisms, i.e., rhizoplane with adhering microorganisms, and the endosphere for root tissues colonized by endophytes (Sánchez-Cañizares et al., 2017) (Figure 1). The rhizosphere and rhizoplane harbor an abundant community of bacteria, archaea, oomycetes and fungi. The collective genome of this microbial community is larger than that of the plant itself, and is often referred to as the plant's second genome (Berendsen et al., 2012). Thus, this alliance of the plant and its associated microorganisms represents a holobiont, which has interdependent, fine-tuned and complex functioning (Berendsen et al., 2012; Vandenkoornhuyse et al., 2015; Sánchez-Cañizares et al., 2017). Bacteria are the most numerous inhabitants of the rhizosphere, so 1g of rhizosphere soil contains approximately 10^8–10^{12} bacterial cells (Kennedy and De Luna, 2005).

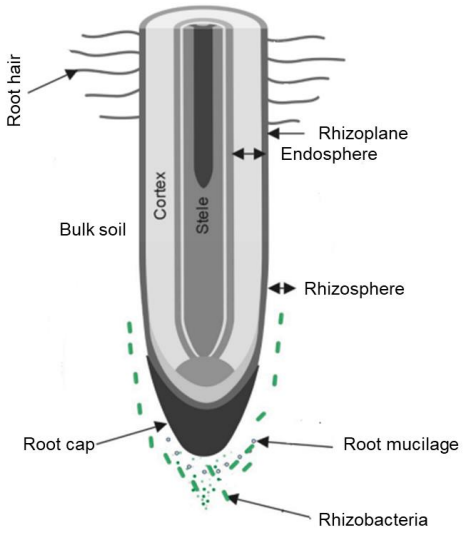

Figure 1. Scheme of microbe-inhabited soil and root compartments, adapted from Hassan et al. (2019).

The "rhizosphere effect", which was first described by Hiltner in 1904 (Hiltner, 1904), explains that plant exudates attract numerous soil microorganisms, so that their number and activity is increased in the rhizosphere. In this system, a plant is a key player, as nearly 40% of all the photosynthates are released directly by roots into the rhizosphere, serving as a fuel for microbial communities, thus recruiting and shaping this microbiome (Berendsen et al., 2012; Tkacz and Poole, 2015). These photosynthates are conditioned by the plant genotype, developmental stage, metabolism, immune system and its ability to exudate (Sánchez-Cañizares et al., 2017). Besides carbon compounds, which represent a rich source of nutrients for this microbial community, plant roots secrete attractants, recognized by microorganisms, after which they start root colonization. Plant root colonization is a key moment in the initiation of: (i) positive interactions, such as communication between plant roots and phytobeneficial microorganisms, (ii) negative interactions, such as root infection by phytopathogens, and (iii) neutral interactions, which have no effect on either participant (Bais et al., 2006). As both phytopathogens and phytobeneficial microorganisms co-habit in the rhizosphere, their interactions largely affect crop productivity and health (Jayaraman et al., 2021).

Positive interactions include those with mycorrhizal fungi, rhizobia, as well as root colonization by phytobeneficial bacteria with biocontrol and/or PGP properties (Vacheron et al., 2013). These bacteria exert numerous beneficial effects on the plant through direct and indirect mechanisms, which enable protection from biotic and abiotic stressors, as well as better seed germination, and root and shoot growth (Glick, 2012). Direct mechanisms employed by beneficial rhizobacteria involve reducing the harmful effects of phytopathogens by affecting its growth/survival. These mechanisms include: (i) antagonism based on the production of different metabolites, (ii) competition with the pathogens for space and nutrients, and (iii) hyperparasitism (Nguvo and Gao, 2019; Morimura et al., 2020). In addition to these mechanisms that affect the pathogen directly, indirect mechanisms, which are mediated by the plant, also come into play. These include induction of plant resistance, as well as mechanisms that increase plant fitness, making it less susceptible to pathogen attack, such as (i) increasing the solubilization of phosphates and nitrogen fixation, (ii) production of siderophores that enable

better absorption of iron, (iii) production of ACC deaminase, thus lowering ethylene levels in plants, as well as (iv) production of phytohormones (Figure 2). Through these indirect mechanisms, beneficial bacteria assist in providing plants with essential nutrients such as phosphorus, nitrogen and iron, and alter levels of phytohormones in plants, thereby mitigating the detrimental effects of environmental stressors (Glick, 2012).

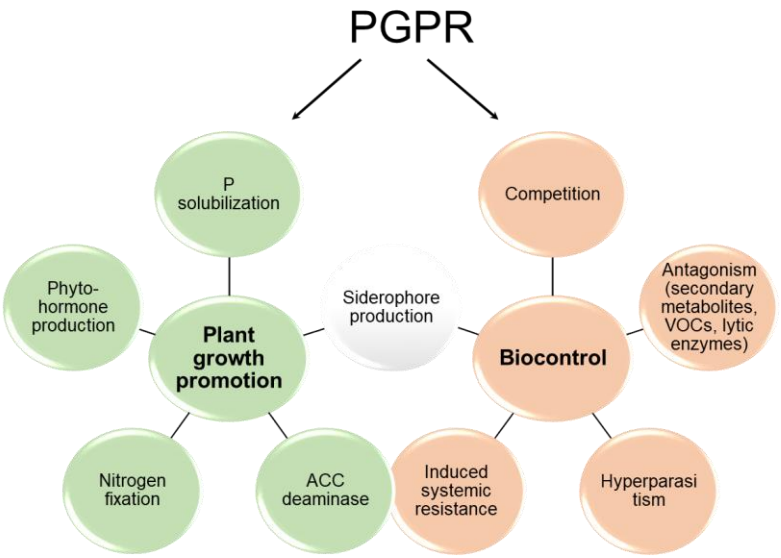

Figure 2. Schematic representation of PGPR plant growth promoting and biocontrol mechanisms.

On the other hand, negative interactions include infection of plant roots with pathogenic bacteria or fungi. Before infecting the host plant tissues, pathogens may grow in the rhizosphere or on the host as saprophytes, managing to escape the rhizosphere battlefield (Raaijmakers et al., 2009). The outcome is directly influenced by the host plant and microbial defense mechanisms, at the level of the holobiont (Berendsen et al., 2012; Vandenkoornhuyse et al., 2015), so upon the pathogen attack, both composition and quantity of root metabolites may change (Rolfe et al., 2019), which can be useful for direct defense against the pathogens (Rizaludin et al., 2021), for signaling the impending threat to the neighboring plants (Pélissier et al., 2021), or for recruiting beneficial microorganisms with biocontrol capabilities. The latter phenomenon is referred to as the 'cry for help' strategy (Rizaludin et al., 2021). By selecting its rhizosphere microbiome, plants contribute to the formation of suppressive soils, where diseases caused by pathogens may be controlled (Tkacz et al., 2015). However, if the pathogen manages to escape from the rhizosphere battlefield, the infection cycle can begin.

2.2. Significance of pathogenic *Fusarium* and soils suppressive to *Fusarium* diseases

Fusarium species occur in soils, but they can also grow in and on living and dead plants (Laraba et al., 2021) and animals (Xia et al., 2019), with the ability to live as pathogens or saprophytes (Smith, 2007; Summerell, 2019). Sometimes, even within the same species (as in the case of *F. oxysporum*), there can be both pathogenic and non-pathogenic strains, distinguished by the presence of pathogenicity-related genes, found on mobile chromosomes (Taylor et al., 2016). *Fusarium* plant-pathogenic species, which are among the most destructive phytopathogens

worldwide, are causing diseases on many agricultural crops (Burgess and Bryden, 2012). This genus exhibits high level of variability in terms of morphological, physiological and ecological properties, which represents a difficulty in establishing a consistent taxonomy of *Fusarium* species (Burgess et al., 1996). An additional difficulty for classification is the existence of both asexual (anamorph) and sexual (teleomorph) phases in their life cycle (Summerell, 2019). However, based on the most widely used classification, the anamorph state of the genus *Fusarium* is classified in the family *Nectriaceae*, order *Hypocreales* and division *Ascomycota* (Crous et al., 2021), and most of the teleomorphs are in the genus *Gibberella*, including the economically important pathogens, such as *G. zeae* (anamorph *F. graminearum*) (Keszthelyi et al., 2007). However, for the sake of taxonomy simplification, the dual anamorph-teleomorph nomenclature for fungi has been abolished, and the name *Fusarium* has been retained for these fungi (Geiser et al., 2013). Diseases caused by *Fusarium* species include blights, wilts and rots of various crops in natural environments and in agroecosystems (Nelson et al., 1994; Ma et al., 2013). *Fusarium* are ubiquitous in parts of the world where cereals and other crops are grown and they produce a wide variety of mycotoxins, which may be present in feed and food products (Babadoost, 2018; Moretti et al., 2018; Chen et al., 2019). Consumption of products that are contaminated with mycotoxins may cause acute or chronic effects in both animals and humans, and could result in immune-suppressive or carcinogenic effects (Jard et al., 2011). There are several mycotoxins produced by *Fusarium* species, such as the trichothecenes deoxynivalenol (DON) and nivalenol (NIV), zearalenone (ZEA), fusaric acid and fusarins (Nešić et al., 2014). By producing mycotoxins and by inducing necrosis and wilting in plants, *Fusarium* fungi are causing substantial economic losses of cereal crops throughout the world (Khan et al., 2017). Their broad distribution has been attributed to their ability to develop on different substrates and plant species, and to produce spores that enable efficient propagation (Desjardins, 2006; Arie, 2019). They are typical soil-borne microorganisms, routinely found in plant-associated fungal communities (Reyes Gaige et al., 2020). Efficient management of plant diseases caused by *Fusarium* is important to limit crop losses and to reduce mycotoxin production in alimentary products (Babadoost, 2018). Because mycotoxin synthesis can occur not only after harvesting but also before, one of the best ways to reduce its presence in food and feed products is to prevent its formation in the crop (Jard et al., 2011). Over the years, different methods, such as the use of resistant cultivars and chemical fungicides, have been undertaken in order to control or prevent crop diseases (Willocquet et al., 2021). In spite of that, *Fusarium* continues to cause enormous crop losses, up to 70% in South America, 54% in the United States and 50% in Europe in the case of Fusarium head blight (FHB) disease of wheat (Scott et al., 2021).

2.2.1. Main control methods available

Fungicides are common agrochemicals used in greenhouse cultivation to protect plants from phytopathogenic infections. However, these chemicals exhibit a low level of specificity, they may have a detrimental effect on the beneficial microbiota, which could all result in adverse effects on soil ecosystems (Mousa and Raizada, 2016). The demethylation inhibitor class of fungicides, consisting of triazoles and imidazoles, is one of the most widely used group of fungicides to suppress *Fusarium* growth. Prothioconazole, a 1,2,4-triazole fungicide, applied before wheat head emergence, can reduce FHB by up to 97% and DON production by 83% (Edwards and Godley, 2010). In contrast, Li and Liu (2022) found that prothioconazole enantiomers increased DON production. However, long-term use of fungicides leads to residual contamination of soils and potentially harmful effects on end users, both animals and humans (Zhang et al., 2020).

Carbendazim and other benzimidazole fungicides used to be very effective against *F. fujikuroi*, *F. proliferatum*, and *F. verticillioides* (formerly *F. moniliforme*), but over time, fungicide-resistant subpopulations of this pathogen have emerged, thus leading to control failures (Chen et al., 2014).

In the case of FHB in wheat, caused by *F. graminearum*, a few resistant cultivars have been described, with different types of resistance taking place (i) during the initial infection, (ii) during the pathogen dissemination within the spike, (iii) during the mycotoxin production, (iv) during kernel infection, as well as (v) resistance that is preventing the yield loss (Chen et al., 2019; Wu et al., 2022). These different types of resistance to FHB are quantifiable, and they are controlled by various quantitative trait loci (QTLs) i.e., more than 550 QTLs located throughout the whole wheat genome (Venske et al., 2019; Fabre et al., 2020). For example, the resistance of the Chinese wheat cultivar Sumai-3 is controlled by the Fhb1 QTL on chromosome 3BS, which is the most stable and efficient resistance locus, allowing a relatively high level of resistance to *Fusarium* spread within the spike (Chen et al., 2019). Although a large number of QTLs have been documented, only few of them have been successfully used to develop resistant varieties (Venske et al., 2019). Besides resistance traits, certain plant varieties possess susceptibility factors, coded by susceptibility (S) genes, that promote pathogen proliferation and disease development (Vogel et al., 2002; Chetouhi et al., 2015; Fabre et al., 2020). In the susceptible wheat cultivar Récital, grain infection by *F. graminearum* does not significantly affect grain development, but affects primary metabolism by altering starch biosynthesis and storage proteins (Chetouhi et al., 2015). Although less studied compared to resistant traits, susceptibility factors may be important in determining the outcome of pathogen attack, opening up the possibility of developing FHB control strategies based on loss of susceptibility genes (Fabre et al., 2020). Besides these, alterations of DNA methylation may be involved in plant defense responses against *Fusarium*, as it has been shown that the removal of cytosine DNA methylation in durum wheat led to an increased resistance to FHB, compared to susceptible, parental lines; this finding may facilitate the development of new, FHB-resistant plant varieties (Kumar et al., 2020).

Transgenic tools have also been proposed to control *Fusarium* diseases, in particular host-induced gene silencing (HIGS). This approach is based on engineering plants to produce interfering RNAs, that are mobile and able to enter fungal cells. Once inside, they trigger the degradation of transcripts of essential genes, such as chitin synthetase and DON-encoding *Tri5* genes. Thus, HIGS has the potential to reduce *Fusarium*-caused diseases under field conditions and to minimize mycotoxin contamination of crops (Cheng et al., 2015). Besides transgenic tools, different methods based on genome editing have been developed. For example, it has been shown that editing the spring wheat genome using the CRISPR machinery may reduce expression of certain genes, for example *TaNFXL1* gene, which is involved in plant resistance, therefore contributing to disease resistance in wheat plants (Brauer et al., 2020). In the following years, it is expected that methods involving genetic engineering (including gene editing) will thrive in efforts to develop resistant varieties with ideal genetic architecture.

Although the best way of coping with mycotoxins would be to prevent their formation in crops, another possibility is to develop post-harvest processes to detoxify already-contaminated feed and food products. The most promising strategies include (i) adsorption, which involves the use of adsorbents that bind mycotoxins in the gastrointestinal system and reduce their absorption and toxicity, (ii) microbial degradation, which involves the removal of the mycotoxins, and (iii) microbial transformation of mycotoxins into less toxic compounds (Awad et al., 2010; Vanhoutte et al., 2016). Hsu et al. (2018) suggested that *Bacillus licheniformis* CK1 could be formulated as a feed additive, due to its ability to adsorb ZEA, and form a ZEA-CK1 complex,

which can then be eliminated through the animal's gastrointestinal system. *Clonostachys rosea* has been shown to degrade ZEA using a zearalenone lactonohydrolase (Kosawang et al., 2014). A soil bacterium of the *Agrobacterium*-*Rhizobium* genus complex converts DON to the less toxic 3-keto DON (Shima et al., 1997). *Burkholderia ambifaria* has the ability to degrade fusaric acid, by using it as the sole source of carbon and nitrogen (Simonetti et al., 2018). However, the applicability of this strategy is not clear in the case of multiple mycotoxin contamination of food and feed, and not all transformations lead to less toxic or non-toxic products (Vanhoutte et al., 2016).

Taken together, the shortcomings of all the control methods adopted so far mandate the need to search for alternative strategies which could provide reliable protection against *Fusarium* diseases – such strategies may have foundation in suppressive soils.

2.2.2. Occurrence of soils suppressive to *Fusarium* diseases

The phenomenon of disease suppressiveness is documented worldwide, and well established in the case of several *Fusarium* pathogenic species (such as *F. culmorum*, *F. oxysporum*, *F. udum*, as well as *F. graminearum*, in a soil fungistasis context), that cause diseases (often wilt, but not exclusively) of various plants (both monocots and dicots) (Table 1). In the Republic of Serbia, however, suppressive soils have not been detected so far, even though plant-beneficial microorganisms have been extensively studied (Jovičić-Petrović et al., 2016; Karličić et al., 2020; Jovičić-Petrović et al., 2021; Kerečki et al., 2022; Dragojević et al., 2023).

Table 1. List of locations with soils suppressive to *Fusarium* diseases known to date, with a pathosystem, disease and the underlying suppression mechanism.

Pathogen	Disease	Country	Suppression mechanism	References
F. culmorum	Seedling blight of barley	Denmark	Soil microbiota that has a more efficient cellulolytic activity	Rasmussen et al., 2002
F. culmorum	*F. culmorum* disease in wheat	Netherlands and Germany	No specific taxa, but a guild of bacteria working together	Ossowicki et al., 2020
F. graminearum	No disease supression tested, only fungistasis	Britanny, France	*Pseudomonas* and *Bacillus*	Legrand et al., 2019
F. oxysporum f. sp. *albedinis*	Bayoud vascular wilt of palm tree	Marocco	Competition with soil microbiota	Rouxel and Sedra, 1989
F. oxysporum f. sp. *melonis*	Fusarium wilt of melon	Châteaurenard, France	Competition with soil microbiota including non-pathogenic *Fusarium*	Louvet et al., 1976; Alabouvette et al., 1985
F. oxysporum f. sp. *fragariae*	Fusarium wilt of strawberry	Korea	*Streptomyces*, wilt-suppressive soil that was developed through monoculture	Cha et al., 2016
F. oxysporum f. sp. *dianthi*	Vascular wilting disease of carnations	Albenga, Italy	Competition with other *Fusarium* strains	Garibaldi et al., 1983
F. oxysporum f. sp. *batatas*	Fusarium wilt on sweet potato	California, USA	No data	Smith and Snyder, 1971
F. oxysporum f. sp. *cubense*	Banana wilt disease	Ayodhya district, India	*Bacillus licheniformis* producing antifungal secondary metabolites	Yadav et al., 2021

F. oxysporum f. sp. *cubense*	Banana wilt disease	Gran Canaria, Spain	Sodium in soil	Domínguez et al., 1996
F. oxysporum f. sp. *cubense*	Banana wilt disease	Indonesia	*Pseudomonas* and *Burkholderia*	Nisrina et al., 2021
F. oxysporum f. sp. *cubense*	Banana wilt disease	Honduras, Costa Rica, Panama and Guatemala	Clay mineralogy, presence of montmorillonite-type clay in suppressive soil	Stotzky and Torrence Martin, 1963
F. oxysporum f. sp. *cubense*	Banana wilt disease	Hainan, China	*Pseudomonas* inducing jasmonate and salicylic acid pathways and shared core microbiome in suppressive soils	Shen et al., 2015; Zhou et al., 2019; Shen et al., 2022; Wang et al., 2022; Lv et al., 2023
F. oxysporum f. sp. *cucumerinum*	Fusarium wilt of cucumber	California, USA	*Pseudomonas* siderophores and lytic bacteria	Sneh et al., 1984
F. oxysporum f. sp. *lini*	Fusarium wilt of flax	California, USA	*Pseudomonas* siderophores	Kloepper et al., 1980
F. oxysporum f. sp. *lini*	Fusarium wilt of flax	Carmagnola and Santena, Italy	Competition with other *Fusarium*	Tamietti and Pramotton, 1990
F. oxysporum f. sp. *lycopersici*	Fusarium wilt of tomato	Noirmoutier, France	Non-pathogenic *F. oxysporum*	Tamietti and Alabouvette, 1986
F. oxysporum f. sp. *lycopersici*	Fusarium wilt of wheat	Albenga, Italy	Non-pathogenic *F. oxysporum* inducing plant defense	Tamietti and Matta, 1984
F. oxysporum f. sp. *lycopersici*	Fusarium wilt of tomato	Albenga, Italy	Non-pathogenic *F. oxysporum* inducing plant defense	Tamietti et al., 1993
F. oxysporum f. sp. *niveum*	Fusarium wilt of watermelon	Florida, USA	Wilt-suppressive soil that was developed through monoculture	Larkin et al., 1993
F. oxysporum f. sp. *radicis-cucumerinum*	Cucumber crown and root rot	Israel	Suppresiveness induced by mixing sandy soil with wild rocket (*Diplotaxis tenuifolia*) debris under field conditions	Klein et al., 2013
F. udum Butl.	Wilt of pigeon-pea	Delhi, India	Soil microbiota	Vasudeva and Roy, 1950

Natural suppressiveness has been extensively studied in the case of *Fusarium* diseases, in particular with the Fusarium wilt suppressive soils of Salinas Valley (California) and Châteaurenard (France). In these soils, plants susceptible to *Fusarium* pathogen(s) show no or limited symptoms and Fusarium wilt disease remains minor despite the long history of cultivation of different crops. In both locations, the small level of disease in plants cannot be attributed to the absence of *Fusarium* in the soil, but rather to plant protection by the soil microbiota (Alabouvette et al., 1984; Sneh et al., 1984). The introduction of small amount of these soils to sterilized suppressive soil or conducive soil significantly decreased Fusarium wilt disease incidence (Scher and Baker, 1980; Alabouvette, 1986). Soil suppressiveness to *Fusarium* diseases is usually natural, but cases of induced suppressiveness are also documented. Thus, soils found in Hainan island (China), that were grown for years with banana in confrontation with pathogenic *F. oxysporum*, displayed rhizosphere enrichment in microbial taxa conferring protection from

banana wilt disease (Shen et al., 2022), watermelon monoculture in Florida induced suppressiveness to wilt caused by *F. oxysporum* f. sp. *niveum* (Larkin et al., 1993), and 15 years of strawberry monoculture in Korea triggered suppressiveness to wilt caused by *F. oxysporum* f. sp. *fragariae* (Cha et al., 2016). Soil addition of wild rocket residues resulted in suppressiveness to cucumber crown and root rot (*F. oxysporum* f. sp. *radicis-cucumerinum*) in Israel (Klein et al., 2013), whereas suppressiveness to Fusarium wilt can also be induced by microbial biofertilizer inoculants reshaping the soil microbiome (Xiong et al., 2017). Thus, organic fertilizer containing *Bacillus amyloliquefaciens* W19 enhanced levels of indigenous *Pseudomonas* and provided suppression of Fusarium wilt of banana (Tao et al., 2020). The combined action of *B. amyloliquefaciens* W19 and *Pseudomonas* is thought to cause a decrease in *Fusarium* density in the root zone of banana. Organic fertilizers inoculated with *Erythrobacter* sp. YH-07 controlled Fusarium wilt in tomato, as a direct result of the bacterial activity, and indirectly by altering the composition of the microbial community (Tang et al., 2023). Organic fertilizers amended with *Bacillus* and *Trichoderma* resulted in an increase in indigenous *Lysobacter* spp., thus indirectly inducing suppression of Fusarium wilt of vanilla (Xiong et al., 2017).

In the case of soils suppressive to *Fusarium* diseases, comparison of suppressive vs. conducive soils has evidenced differences in the occurrence or prevalence of multiple taxa. No single phylum was uniquely associated with *F. oxysporum* wilt suppressiveness in Korean soils, even though *Actinobacteria* was identified as the most prevalent bacterial taxa colonizing strawberry in suppressive soils (Cha et al., 2016). Likewise, the bacterial genera *Devosia*, *Flavobacterium* and *Pseudomonas* were more abundant (and the pathogen less abundant) in Chinese soils suppressive to banana wilt than in non-suppresive soils, and *Pseudomonas* inoculants isolated from suppressive soils could control the disease (Lv et al., 2023). Compared with conducive soil, Fusarium wilt suppressive soil from Châteaurenard displayed higher relative abundance of *Adhaeribacter*, *Arthrobacter, Amycolatopsis*, *Geobacter, Massilia*, *Microvirga*, *Paenibacillus*, *Rhizobium*, *Rhizobacter*, *Rubrobacter* and *Stenotrophomonas* (Siegel-Hertz et al., 2018). However, differences were also found in the fungal community, with several fungal genera (*Acremonium*, *Ceratobasidium*, *Chaetomium*, *Cladosporium*, *Clonostachys*, *Mortierella*, *Penicillium*, *Scytalidium*, *Verticillium*, but also *Fusarium*) detected exclusively in the Fusarium wilt suppressive soil (Siegel-Hertz et al., 2018). Data also pointed to a greater degree of microbial complexity in suppressive soils, with particular co-occurrence networks of taxa (Bakker et al., 2014; Lv et al., 2023). In German and Dutch soils, co-occurrence networks showed that the suppressive soil microbiota involves a guild of bacteria – a functional group which was dominated by *Acidobacteria* in two of four suppressive soils (Ossowicki et al., 2020).

Fungistasis can also affect *Fusarium* pathogens (de Boer et al., 2019; Legrand et al., 2019), but its significance in relation to different *Fusarium* species needs clarification. In the work by Legrand et al. (2019), soil fungistasis was tested by quantifying growth of *F. graminearum* in autoclaved vs. non-autoclaved soils using quantitative PCR (qPCR) approach after an incubation period. It was shown that *F. graminearum* growth was significantly reduced in non-autoclaved soils compared to the autoclaved soils, and moreover, in non-autoclaved soils there was a gradient in the *F. graminearum* DNA quantity, suggesting different levels of soils' fungistasis (Legrand et al., 2019). The results also highlighted higher bacterial diversity, a higher prevalence of *Pseudomonas* and *Bacillus* species and a denser network of co-occurring bacterial taxa in soils with fungistasis. These data suggest the importance of cooperation within bacterial communities to control *F. graminearum* in soil (Legrand et al., 2019).

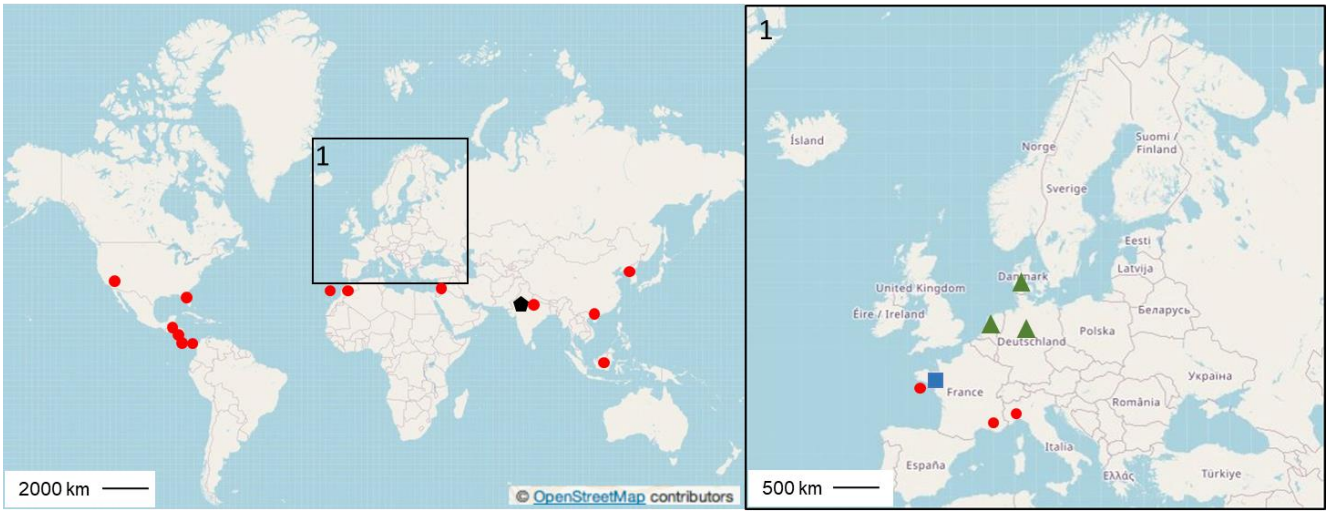

Figure 3. Geographic location of European field locations (map on the right) with soils suppressive to *Fusarium* diseases, i.e., in France (Noirmoutier island, Châteurenard in South-East France, and Brittany), Denmark, The Netherlands, Germany, Italy (Albenga, Carmagnola and Santena) and Gran Canaria Island (Spain, located in the Atlantic ocean), with the corresponding pathogen i.e., *Fusarium oxysporum* (red dot), *Fusarium culmorum* (green triangle), or *Fusarium graminearum* (blue square). Soils suppressive to *Fusarium oxysporum* were also found outside Europe (map on the left), i.e., in North America: in California and Florida; Central America: in Honduras, Costa Rica, Panama and Guatemala; in Asia: in Korea, China, India, Israel and Indonesia; and in Africa: in Marocco. Soils suppressive to *Fusarium udum* were found in India (black pentagon). Adapted from Todorović et al. (2023b).

It appears that suppressiveness to *Fusarium* diseases has been studied and occurs in numerous parts of the world (Figure 3). However, many studies focused on a few, geographically-close soils, which does not provide a global view on the importance of microbial diversity. Two studies have considered geographically diverse agricultural soils suppressive to Fusarium wilt. Various Chinese soils suppressive to banana wilt mediated by *F. oxysporum* were shown to share a common core microbiota, specific to suppressive soils, which included the genus *Pseudomonas* (Shen et al., 2022). In a wider range of soils from the Netherlands and Germany, soils suppressive to *F. culmorum*-mediated wilt of wheat did not display a specific bacterial species that correlated with suppressiveness (Ossowicki et al., 2020). There was no relation either with soil physicochemical composition (i.e., soil type, pH, contents in C, N, or bioavailable Fe, K, Mg, P) or field history, yet suppressiveness was microbial in nature, as sterilizing suppressive soils made them become conducive. Taken together, this might be explained by the fact that protection of wheat from *F. culmorum*-mediated wilt corresponds to a case of natural suppressiveness, where biogeographic patterns are probably important, whereas banana wilt disease-suppressive soils are induced by monoculture, with convergent effects resulting from similar banana recruitment across different soil types. However, more research is needed in order to better understand microbial diversity patterns in soils with natural and induced suppressiveness.

2.2.3. Effects of farming practices on soils suppressive to *Fusarium* diseases

As many other soil-inhabiting pathogenic fungi, *Fusarium* can overwinter as mycelium in plant debris or dormant structures in the soil, which causes the initial infection of plants in the following season (Leplat et al., 2013; Xu et al., 2021). Therefore, cultural practices involving the removal of the overwintering inoculum of the pathogen from soils are useful to prevent future infection (Voigt, 2002). However, farming practices also influence soil suppressiveness by shaping the rhizosphere microbial community (Campos et al., 2016) and stimulating the activity of beneficial rhizosphere microorganisms (Janvier et al., 2007). In this context, various agricultural practices, such as crop rotation/monocropping, organic amendments, tillage and fertilizers, are important considerations when developing suppressiveness-based control methods in farm fields (Janvier et al., 2007).

In rare cases, crop monoculture may induce suppressiveness to *Fusarium* diseases by leading to an increase in indigenous plant-beneficial microbiota, usually after a disease outbreak (Larkin et al., 1993; Weller et al., 2002; Shen et al., 2022). However, cropping systems based on rotation of different plant species usually result in reduced survival of soil-borne pathogen propagules over the short term (Winter et al., 2014). Crop rotation may reduce severity and incidence of diseases caused by *Fusarium* (Wang et al., 2015; Khemir et al., 2020). For example, compared with the tomato monoculture, soil management under wheat-tomato rotation changes soil microbial composition by increasing the abundance of microbial taxa such as *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*, *Aspergillus*, *Penicillium* and *Mortierella*, which may control Fusarium wilt of tomato (De Corato et al., 2020). Reduced incidence of *F. pseudograminearum* and *F. culmorum*-caused diseases in the soils under cereal–legumes rotation management may be due to the non-host character of the legumes (Evans et al., 2010). However, not all crop rotations lead to reduced disease pressure (Ranzi et al., 2017). In the case of the FHB, it was initially advocated to rotate wheat and maize with crops like soybean, until it was shown that *F. graminearum* can also cause disease in soybean, as it has a wide range of hosts (Marburger et al., 2015). This suggests that there is a lack of clear correlation between crop rotation and *Fusarium* disease incidence.

Crop residues of high cellulose content promoted the activity of beneficial cellulolytic microorganisms and limited the development of *F. culmorum* (Rasmussen et al., 2002), as organic amendments represent a favorable environment for beneficial microorganisms that are able to combat phytopathogenic *Fusarium* species (Maher et al., 2008; Cuesta et al., 2012). Thus, soils with added organic amendments exhibited inhibitory effects against *F. verticillioides* by reducing the production of fungal pigment and sporulation, consequently disabling fungal spread (Nguyen et al., 2018). Addition of vermicompost reduced tomato infection by *F. oxysporum* f. sp. *lycopersici* (FOL) (Szczech, 1999) and mulched straw contributed to the suppression of seedling blight caused by *F. culmorum* (Knudsen et al., 1999). Soils supplemented with coffee residue compost or rapeseed meal exhibited suppressiveness to *F. oxysporum*-mediated wilt, and microorganisms isolated from supplemented soils inhibited *F. oxysporum* growth on agar plates (Mitsuboshi et al., 2018). Carbon addition to soil influenced the soil microbiome, enhancing *Fusarium*-inhibitory populations from the *Streptomyces* genus (Dundore-Arias et al., 2020). One study tested the effects of 18 composts (made from different mixtures of manure, domestic biowaste and green waste) on Fusarium wilt disease suppression, caused by *F. oxysporum* f. sp. *lini*, demonstrating that only one compost did not positively affect the disease suppression (Termorshuizen et al., 2006). The efficiency of organic amendments in controlling plant diseases is determined by the

pathosystem, the application rate, the kind of amendment and the level of maturity of composts or disintegration phase of crop residues (Janvier et al., 2007).

Tillage, which is one factor influencing organic matter decomposition, appears to have contrasting effects on soil suppressiveness. Under conventional tillage, tillage depth appears to play a crucial role in soil survival of *Fusarium*, such that the deeper the tillage, the lower the abundance of *Fusarium* species (Steinkellner and Langer, 2004). This can be partly explained by the fact that the pathogen is displaced from its niche, reducing its ability to survive (Bailey and Lazarovits, 2003), and the rate of decomposition of buried residues is faster than at the soil surface (Leplat et al., 2013). The carbon released during these decomposition processes increases the activity of the soil microbiota, thereby improving the overall functioning of the soil (Bailey and Lazarovits, 2003). Under conservation tillage, surface residues persist and can act as a long-term source of inoculum for plant infection by *F. verticillioides*, *F. proliferatum* and *F. subglutinans*, as they can colonize crop residues and produce spores that often survive the period when plants are absent from the agrosystem (Bockus and Shroyer, 1998; Cotten and Munkvold, 1998; Pereyra et al., 2004). This is consistent with results suggesting that conservation tillage and leaving crop residues *in situ* increase *Fusarium* abundance (Govaerts et al., 2008; Wang et al., 2020a). For example, spores of *Fusarium* species can be recovered from plant residues more than two years after harvest (Pereyra et al., 2004). However, in certain cases, lower occurrence of plant infection by *F. culmorum*, *F. equiseti* (Weber et al., 2001) and *F. pseudograminearum* (Theron et al., 2023) was detected under conservation tillage compared with conventional tillage. These contrasting results might be due to differences in environmental factors, cropping patterns and soil types, which could modulate interactions between soil conditions, *Fusarium* ecology and plant physiology (Sturz and Carter, 1995). The use of simplified tillage practices was proposed to reduce *F. culmorum* abundance, by mixing crop residues with the topsoil layer to promote the growth of beneficial straw-decomposing microorganisms (Weber and Kita, 2010).

Concerning fertilizers, it has generally been shown that nitrite reduced the population of *F. oxysporum* (Löffler et al., 1986) and that the addition of phosphorus fertilizer, significantly reduced *F. oxysporum*-caused wilting in chickpea, lentil and lupine (Elhassan et al., 2010). Organic fertilizers can lead to an increase in indigenous microbial populations, thus contributing to suppression of Fusarium wilt disease (Montalba et al., 2010; Raza et al., 2015). When grown with the addition of organic N fertilizer, highbush blueberry exhibited increased tolerance to *F. solani*, in parallel to increased soil microbial activity and mycorrhizal colonization (Montalba et al., 2010).

2.2.4. *Fusarium graminearum*

F. graminearum is the fourth-ranked fungal phytopathogen in terms of economic importance (Dean et al., 2012; Legrand et al., 2017). It is distributed worldwide (Babadoost, 2018), and it is responsible for rotting, necrosis, kernel damage and mycotoxin production (Ma et al., 2013) in small grain cereals, such as wheat, barley, rice and oats (Goswami and Kistler, 2004). Wheat, a worldwide staple food and an important cereal for human civilization, is severely affected by *F. graminearum* diseases. This fungus causes wheat yield losses of 20% to 70%, and contaminates the wheat kernels with mycotoxins, therefore causing additional economic losses (Bai and Shaner, 1994). *F. graminearum* produces spores which enable efficient propagation, i.e., sexual spores (ascospores), as well as two types of asexual spores, i.e., (i) macroconidia, which are often found on the surface of diseased plants, and (ii) chlamydospores (survival structures), which are thick walled and produced from macroconidia or older mycelium (Leslie and Summerell, 2006;

Ma et al., 2013). In Serbia, agroecological conditions are favorable for the development of phytopathogenic and toxicogenic *F. graminearum* (Obradović et al., 2017).

Plant infection by *F. graminearum* occurs in few successive stages (Figure 4) and diseases caused by this fungal pathogen include damping-off, root and crown rot, and FHB in small grain cereals (Besset-Manzoni et al., 2019). This pathogen grows saprophytically on crop debris, which represents an overwintering reservoir of the pathogen (Brown et al., 2010). Seeds infected with *F. graminearum* in the previous season can also serve as disease initiators (Jiménez-Díaz et al., 2015). Sometimes, seed infection by *F. graminearum* may lead to damping-off disease, which is manifested as reduced seed germinability and post-emergence seedling blight (Dal Bello et al., 2002). Soil-borne inoculum of *F. graminearum* may also infect roots and cause damage to the collar (Ares et al., 2004), causing root and crown rot. Symptoms of root and crown rot include browning and rotting of roots, crowns, and lower stem tissues, followed by necrosis (Fernandez and Conner, 2011; Taheri, 2018). During the crop anthesis and under warm and humid weather conditions, asexual conidia, sexual ascospores or chlamydospores are dispersed by rain or wind and reach the outer anthers and outer glumes of the plant. After spore germination, hyphae penetrate the host plant through the cracked anthers, followed by inter- and intracellular mycelial growth, resulting in damage to host tissues and especially causing the FHB (Brown et al., 2010). Typical symptoms of FHB begin soon after flowering, as diseased spikelets gradually bleach, leading to bleaching of the entire head. After this stage, black spherical structures called perithecia may appear on the surface of diseased spikelets. Later, as the disease becomes more severe, the fungus begins to attack the kernels inside the head, causing them to wrinkle and shrink (Schmale and Bergstrom, 2003).

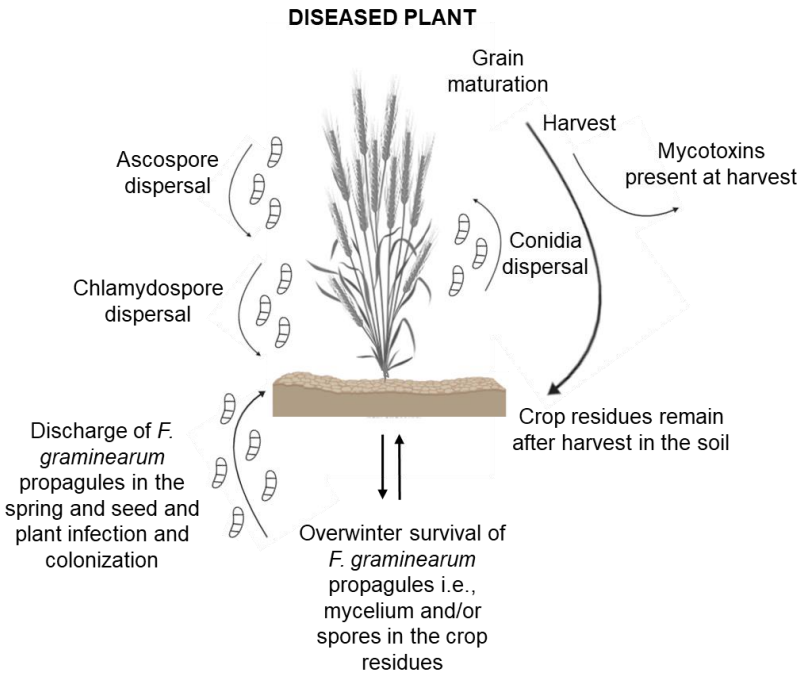

Figure 4. Life cycle of *Fusarium graminearum.* Adapted from Todorović et al. (2023b).

Besides damaging the plants and causing substantial yield losses, *F. graminearum* also contaminates them with mycotoxins. *F. graminearum* produces several types of mycotoxins, such as trichothecenes DON and NIV, ZEA and fusarins (Nešić et al., 2014). It has been reported that

trichothecenes even play a key role in pathogenesis and that the aggressiveness of *Fusarium* depends on its DON and NIV-producing capacity (Mesterházy, 2002). DON production by *F. graminearum* has been reported as essential for disease development in wheat spikes (Cuzick et al., 2008), while spikes treated with DON or NIV led to yield losses even in the absence of the pathogen, indicating a strong negative effect of these mycotoxins on wheat growth (Ittu et al., 1995). In order to prevent yield losses and cereal mycotoxin contamination due to *F. graminearum*, biological control seems to be a promising solution. Several bacterial species were found to have an antagonistic effect on this fungal pathogen, including *Pseudomonas*, *Bacillus*, *Brevibacillus*, *Chryseobacterium*, *Kosakonia* and *Burkholderia,* through different modes of action (Petti et al., 2010; Tyc et al., 2015; Chen et al., 2018a; Xu et al., 2020; Masri et al., 2021; Singh et al., 2021). Soils suppressive to diseases caused by this pathogen may provide a clue on the functioning of these soils and indigenous microbial community, however, to the author's knowledge, soils suppressive to *F. graminearum* diseases have only been identified in the soil fungistasis context so far (Legrand et al., 2019).

2.3. Biocontrol agents against *Fusarium* and their modes of action

Plant-beneficial microorganisms present in the rhizosphere may protect plants from *Fusarium* pathogens, through different modes of action, including: (i) antagonism based on the production of different metabolites, i.e., secondary metabolites, VOCs (Volatile Organic Compounds) and enzymes, (ii) competition with the pathogens for space and nutrients, (iii) hyperparasitism or (iv) induction of resistance in the plant (Figure 5) (Nguvo and Gao, 2019; Morimura et al., 2020). Some of them are also able to inhibit mycotoxin synthesis or to enhance their detoxification (Legrand et al., 2017; Morimura et al., 2020). Certain biocontrol microorganisms have multiple modes of action, which may be expressed simultaneously or sequentially (Legrand et al., 2017).

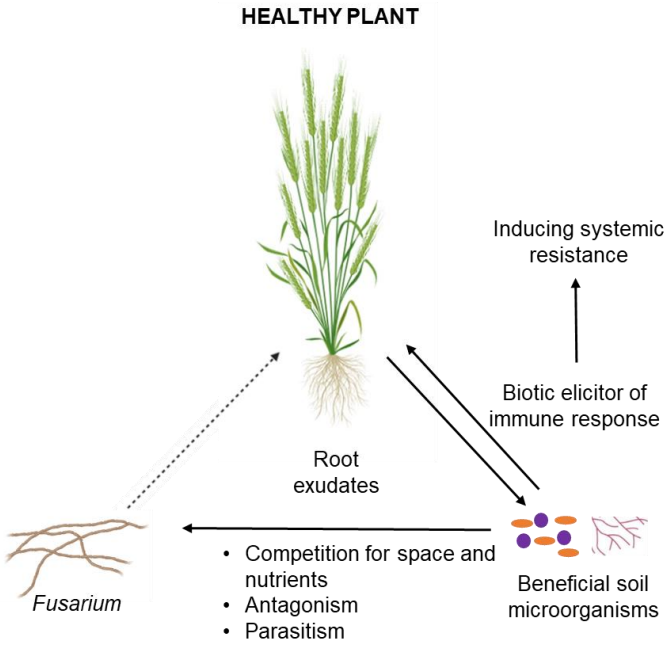

Figure 5. Dynamic interactions between beneficial soil microorganisms, plant and phytopathogenic *Fusarium* species. Adapted from Todorović et al. (2023b).

When searching for potential biocontrol agents, a logical choice would be to explore the pathogen and/or disease-suppressing habitats with high microbial biomass. For example, such habitats include suppressive compost (Pugliese et al., 2011; De Corato, 2020), healthy plants grown in suppressive soils (where the pathogen is present), fungistatic or suppressive soils (Köhl et al., 2011). So far, there are biocontrol strains that originate from suppressive soils, and they have been investigated as means to understand disease suppressiveness. In the case of *Fusarium* diseases, examples include *Pseudomonas* sp. Q2-87 (*P. corrugata* subgroup) (Weller et al., 2007), isolated from wheat in take-all decline soils, but that protects tomato from *Fusarium oxysporum* f. sp. *radicis-lycopersici, Pseudomonas* sp. C7 (*P. corrugata* subgroup) (Lemanceau and Alabouvette, 1991) and non-pathogenic *F. oxysporum* Fo47, both isolated from soils suppressive to Fusarium wilt disease of tomato, in Châteurenard (Fuchs et al., 1997, 1999; Duijff et al., 1998; Zhang et al., 2018b). Efficient biocontrol strains are able to restrict pathogen growth and/or development by different mechanisms, while the most successful ones exhibit multiple modes of action. For example, *Pseudomonas* sp. Q2-87 produces an antifungal metabolite 2,4-diacetylphloroglucinol (DAPG) (Weller et al., 2007), while *Pseudomonas* sp. C7 and *F. oxysporum* Fo47 are better competitors for nutrients than pathogenic *Fusarium* (Lemanceau and Alabouvette, 1991; Zhang et al., 2018b).

2.3.1. Antagonism

An important microbial mechanism to suppress plant pathogens is the secretion of various antifungal metabolites. They include antifungal secondary metabolites, sometimes termed antibiotics (e.g., fengycin, iturin, surfactin (Chen et al., 2018a), fusaricidin and polymyxin (Zalila-Kolsi et al., 2016), DAPG (coded by the *phlABCD* gene cluster; Bangera and Thomashow, 1999; Kang, 2012), pyrrolnitrin (coded by the *prnABCD* gene cluster; Kirner et al., 1998; Huang et al., 2018), phenazine(s) (coded by the *phzABCDEFG* gene cluster; Dar et al., 2020; Xu et al., 2020)), as well as VOCs (Zaim et al., 2016; Legrand et al., 2017) (Table 2). Extracellular lytic enzymes such as cellulase, chitinase, pectinase, xylanase (Khan et al., 2018), protease and glucanase (Saravanakumar et al., 2017) can also interfere with *Fusarium* growth or activity (Table 2). Besides these enzymes, it is known that there are other enzymes produced by microbes that can break down complex carbohydrates, such as gluconjugates degrading enzymes, cellobiases and xylobiases, peptidoglucanases, caragenases and agarases, lytic polysaccharide monoxygenases, arabinogalactanases, mannanases and xyloglucanases (López-Mondéjar et al., 2022). All of these enzymes are classified in several groups, i.e., glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs) and auxiliary activities (AAs), according to the type of reaction that they catalyze, and each consisting of several families (Carbohydrate Active Enzymes database; http://www.cazy.org/; Drula et al., 2022). For example, GH group consists of families involved in breaking down the glycosidic bonds in complex carboxydrates, such as cellulose, chitin, xylan, mannans, glucans, etc. (Henrissat, 1991), and AA group includes ligninolytic enzymes and lytic polysaccharide monoxygenases (with AA10 family potentially targeting chitin) (Drula et al., 2022).

Table 2. Biocontrol agents, *Fusarium* pathogens and biocontrol enzymes and metabolites.

Biocontrol agent	Pathogen	Biocontrol enzymes and metabolites	References
Bacillus subtilis	*F. oxysporum* *F. graminearum*	Cellulase, chitinase, pectinase, xylanase, protease, fengycins and surfactins	Zhao et al., 2014; Zalila-Kolsi et al., 2016; Khan et al., 2018
Bacillus velezensis	*F. graminearum* *F. culmorum*	Fengycin B, iturin A, surfactin A and siderophores	Chen et al., 2018; Adeniji et al., 2019
Bacillus pumilus	*F. oxysporum*	Chitinolytic enzymes and antibiotic surfactin	Agarwal et al., 2017
Bacillus amyloliquefaciens	*F. graminearum*	Iturin and surfactin	Zalila-Kolsi et al., 2016
Brevibacillus fortis	*F. oxysporum*	Edeine	Johnson et al., 2020
Brevibacillus reuszeri	*F. oxysporum*	Chitinolytic enzymes	Masri et al., 2021
Burkholderia sp.	*F. oxysporum*	Phenazine-1-carboxylic acid	Xu et al., 2020
Chryseobacterium sp.	*F. solani*	VOCs	Tyc et al., 2015
Gluconacetobacter diazotrophicus	*F. oxysporum*	Antibiotic (pyoluteorin) and VOCs	Logeshwarn et al., 2011
Kosakonia arachidis	*F. verticillioides* *F. oxysporum*	Chitinase, protease, cellulase and endoglucanase	Singh et al., 2021
Lysobacter antibioticus	*F. graminearum*	VOCs	Kim et al., 2019
Paenibacillus polymyxa	*F. graminearum* *F. oxysporum*	Cell-wall degrading enzymes, fusaricidin, polymyxin and VOCs	He et al., 2009; Raza et al., 2015; Zalila-Kolsi et al., 2016
Paenibacillus polymyxa	*F. oxysporum*	VOC (Hydrogen cyanide)	Xu and Kim, 2014
P. chlororaphis	*F. graminearum*	Pyrrolnitrin	Huang et al., 2018
Pseudomonas sp.	*F. verticillioides* *F. graminearum*	Antifungal antibiotics and fluorescent pigments	Pal et al., 2001
Pseudomonas sp.	*F. oxysporum*	DAPG	Kang, 2012
Streptomyces spp.	*F. oxysporum*	Antibiotic compounds, lipopeptin A and lipopeptin B	Cuesta et al., 2012; Wang et al., 2023
Trichoderma sp.	*F. oxysporum* *F. caeruleum*	Pyrones, koningins and viridins	Reino et al., 2008

Pseudomonadota representatives (formerly *Proteobacteria*) are known for disturbing *Fusarium* growth or activity. Thin layer chromatography analysis showed that *Gluconacetobacter diazotrophicus* produces pyoluteorin, which is involved in the suppression of *F. oxysporum* (Logeshwarn et al., 2011), while *Burkholderia* sp. HQB-1 produces phenazine-1-carboxylic acid, which is efficient at controlling Fusarium wilt of banana, caused by *F. oxysporum* f. sp. *cubense* (Xu et al., 2020). DAPG-producing *Pseudomonas* sp. NJ134 was successful in suppressing Fusarium wilt caused by FOL (Kang, 2012). *Pseudomonas* sp. EM85 was able to suppress disease caused by *F. verticillioides* and *F. graminearum*, by producing antifungal antibiotics and fluorescent pigments (Pal et al., 2001). *P. chlororaphis* G05 inhibited *F. graminearum* mycelial growth and germination of conidia by producing pyrrolnitrin (Huang et al., 2018). *Bacillota* representatives (formerly *Firmicutes*), i.e., *Bacillus* and *Brevibacillus* species, are highlighted in several studies as candidates for *Fusarium* biocontrol through production of antifungal metabolites (Palazzini et al., 2007; Zhao et al., 2014; Chen et al., 2018a; Johnson et al., 2020). *Brevibacillus fortis* NRS-1210 produces edeine, a compound with antimicrobial activity, which inhibits chlamydospore germination and conidia growth in *F. oxysporum* f. sp. *cepae* (Johnson et al., 2020). *Bacillus subtilis* SG6 has the ability to produce fengycins and surfactins acting against *F. graminearum* (Zhao et al., 2014), whereas *Bacillus velezensis* LM2303 exhibited strong antagonistic activity against *F. graminearum* and significantly reduced FHB severity under field conditions (Chen et al., 2018a). Genome mining of *B. velezensis* LM2303 identified 13 biosynthetic gene clusters encoding secondary metabolites and chemical analysis confirmed their presence. These metabolites included three antifungal metabolites (fengycin B, iturin A, and surfactin A) and eight antibacterial metabolites (surfactin A, butirosin, plantazolicin and hydrolyzed plantazolicin, kijanimicin, bacilysin, difficidin, bacillaene A and bacillaene B, 7-o-malonyl macrolactin A and 7-o-succinyl macrolactin A) (Chen et al., 2018a). Besides bacteria, *Trichoderma* fungi synthesize a number of secondary metabolites such as pyrones (which completely inhibit spore germination of *F. oxysporum*), koningins (which affect the growth of *F. oxysporum*) and viridin (which prevents the germination of spores of *F. caeruleum*) (Reino et al., 2008). In soils suppressive to Fusarium wilt of strawberry, in Korea, soil suppressiveness was a result of enrichment of *Streptomyces* that produce antifungal secondary metabolite named thiopeptide (Cha et al., 2016), while phenazine-producing *Pseudomonas* contributed to soil suppressivenes to Fusarium wilt in Châteurenard (Mazurier et al., 2009).

VOCs have recently received more attention, as they can enable interactions between organisms in the soil ecosystem through both water and air phases (de Boer et al., 2019). *Paenibacillus polymyxa* WR-2 produced VOCs when cultivated in the presence of organic fertilizer and root exudates. Among them, benzothiazole, benzaldehyde, undecanal, dodecanal, hexadecanal, 2-tridecanone and phenol inhibited mycelial growth and spore germination of *F. oxysporum* f. sp. *niveum* (Raza et al., 2015). Similarly, hydrogen cyanide (HCN)-producing *P. polymyxa* SC09-21 suppressed Fusarium crown and root rot disease of tomato, caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*, for 80%, under the greenhouse conditions (Xu and Kim, 2014). *Chryseobacterium* sp. AD48 inhibited growth of *F. solani* through the production of VOCs (Tyc et al., 2015). Du et al. (2022) found that acetoin and 2-heptanol, produced by *B. subtilis*, reduce Fusarium crown and root rot disease in tomato. VOCs produced by *Lysobacter antibioticus* HS124 enhanced mycelial development, but they simultaneously reduced sporulation and spore germination of *F. graminearum* (Kim et al., 2019). In addition, testing the antagonistic mechanisms of *Aspergillus pseudocaelatus* and *Trichoderma gamsii* revealed the presence of the VOCs 2,3,4-trimethoxyphenylethylamine, 3-methoxy-2-(1-methylethyl)-5-(2-methylpropyl) pyrazine, (Z)-9- octadecenamide, pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-

(2-methylpropyl)-, thieno [2,3-c] pyridine-3-carboxamide,4,5,6,7-tetrahydro-2-amino-6-methyl- and hexadecanamide, which have an inhibitory activity against *F. solani* (Zohair et al., 2018).

Regarding extracellular lytic enzymes, *B. subtilis* 30VD-1 antagonized FOL by producing cellulase, chitinase, pectinase, xylanase and protease (Khan et al., 2018), while *Bacillus pumilus* synthesized a chitinolytic enzyme that reduced severity of disease caused by *F. oxysporum* on buckwheat under gnotobiotic conditions (Agarwal et al., 2017). *Brevibacillus reuszeri* inhibited growth of *F. oxysporum* by producing chitinolytic enzymes (Masri et al., 2021). *Kosakonia arachidis* EF1 produced different cell-wall degrading enzymes, such as chitinases, proteases, cellulases and endoglucanases, which inhibited growth of *F. verticillioides* and *F. oxysporum* f. sp. *cubense*. Scanning electron microscopy revealed broken fungal mycelia surface and hyphae fragmentation when pathogens were grown in the presence of *K. arachidis* EF1 (Singh et al., 2021). In the case of *F. graminearum*, two *P. polymyxa* isolates, W1-14-3 and C1-8-b, both producers of fungal cell-wall degrading enzymes, reduced the severity of FHB, caused by this fungal pathogen, by 56,5% and 55,4 %, respectively (He et al., 2009). The role of extracellular lytic enzymes in suppressive soils can be significant, as soil microbiota may protect barley from *Fusarium culmorum*-caused disease, via a more efficient cellulolytic activity than the pathogen, which is, consequently, outcompeted for nutrients (Rasmussen et al., 2002). In soils suppressive to banana wilt disease in China, suppressiveness results in part from chitinolytic effects of the soil microbiota against the pathogen (Lv et al., 2023).

2.3.2. Competition for space and nutrients

In the case of competition, biocontrol of pathogens occurs when another microorganism is able to colonize the environment faster and use nutrient sources more efficiently than the pathogen itself, especially under limiting conditions (Maheshwari, 2013; Legrand et al., 2017). Bacteria and fungi have the ability to compete with *Fusarium*, but the underlying mechanisms of competition are sometimes unclear. Competition may involve bacteria such as *Pseudomonas capeferrum* (ex *putida*) strain WCS358, which suppresses Fusarium wilt of radish by competing for iron through the production of its pseudobactin siderophore (Lemanceau et al., 1993). In *P. putida* (Trevisan) Migula isolate Corvallis, competition for root colonization entails plant's production of agglutinin, and *P. putida* mutants lacking the ability to agglutinate with this plant glycoprotein showed reduced levels of rhizosphere colonization and suppression of Fusarium wilt of cucumber (Tari and Anderson, 1988). In addition to bacteria, the fungus *Trichoderma asperellum* strain T34 can control the disease caused by FOL on tomato plants by competing for iron (Segarra et al., 2010), while a non-aflatoxigenic *Aspergillus flavus* strain was found to outcompete a mycotoxin-producing *F. verticillioides* during colonization of maize (Reis et al., 2020).

In the case of soils suppressive to *Fusarium* diseases, competition with pathogenic *Fusarium* species is considered important, involving the entire soil microbiota or, more specifically, non-pathogenic *Fusarium* strains in Châteaurenard soils (Louvet et al., 1976; Alabouvette, 1986) and fluorescent *Pseudomonas* (iron competition) in soils of Salinas Valley (Sneh et al., 1984).

2.3.3. Hyperparasitism

Mycoparasitism is a lifestyle, dating back to more than 400 million years ago, during which one fungus parasitizes another fungus (Kubicek et al., 2011). It involves direct physical contact with the host mycelium (Pal and McSpadden Gardener, 2006), secretion of cell wall-degrading

enzymes and subsequent hyphal penetration (Viterbo et al., 2002). Mycoparasitic relationships can be biotrophic, where the host remains alive and the mycoparasitic fungus obtains nutrients from the mycelium of its partner, or necrotrophic, where the parasite contacts and penetrates the host, resulting in the death of the host and allowing the mycoparasite to use the remains of the host as a nutrient source (Jeffries, 1995). Several species of fungi are mycoparasitic, of which *Trichoderma* is the best described. Contact between the mycoparasitic fungi *Gliocladium roseum*, *Penicillium frequentans*, *T. atroviride*, *T. longibrachiatum* or *T. harzianum* and their phytopathogenic targets *F. culmorum*, *F. graminearum* and *F. nivale* triggers the formation of various mycoparasitic structures, such as hooks and pincers, which lead to cell disruption in the phytopathogens (Pisi et al., 2001; Karličić et al., 2021). When *T. asperellum* and *T. harzianum* were grown in the presence of *F. solani* cell wall, they secreted several cell wall-degrading enzymes, such as β-1,3-glucanase, *N*-acetylglucosaminidases, chitinase, acid phosphatase, acid proteases and alginate lyase (Qualhato et al., 2013), and similarly, *Clonostachys rosea* produced chitinase and β-1,3-glucanase in the presence of *F. oxysporum* cell wall (Chatterton and Punja, 2009). *Sphaerodes mycoparasitica* is a biotrophic fungus that parasitizes *F. avenaceum*, *F. oxysporum* and *F. graminearum* hyphae and forms hooks as parasitic structures (Vujanović and Goh, 2009). However, the direct contribution of mycoparasitism to biological control is difficult to quantify, as mycoparasitic fungi typically exhibit a number of different biocontrol mechanisms (Pal and McSpadden Gardener, 2006).

2.3.4. Induced systemic resistance

Induced systemic resistance (ISR) is the phenomenon whereby a plant, once appropriately stimulated by biological or chemical inducers, exhibits enhanced resistance when challenged by a pathogen (Walters et al., 2013). ISR in plants is usually activated via signaling pathways regulated by jasmonate and ethylene, and in certain cases by salicylic acid, pyoverdin, and/or cyclic lipopeptides surfactants (Gamalero and Glick, 2011). ISR involves (i) the plant perception of inducing signals, (ii) signal transduction by plant tissues, and (iii) expression of plant mechanisms inhibiting penetration of the pathogen into the host tissues (Magotra et al., 2016). A wide variety of microorganisms, including the bacteria *Pseudomonas, Bacillus, Streptomyces* and the fungi *Trichoderma* and non-pathogenic *F. oxysporum* can induce ISR (Fuchs et al., 1997; Choudhary et al., 2007; Zhao et al., 2014; Galletti et al., 2020) in plants against *Fusarium* (Table 3). ISR in the plant-*Fusarium* system is based on microbial induction of the activity of various defense-related enzymes in plants, such as chitinase (Amer et al., 2014), lipoxygenase (Aydi Ben Abdallah et al., 2017), polyphenol oxidase (Akram et al., 2013), peroxidase, phenylalanine ammonia-lyase (Zhao et al., 2012), β-1,3-glucanase, catalase (Sundaramoorthy et al., 2012), and also the accumulation of phytoalexins, defense metabolites against fungi (Kuć, 1995). Cyclic lipopeptide antibiotics, e.g., fusaricidin (Li and Chen, 2019) and surfactin (Chen et al., 2018a), external cell components, e.g., lipopolysaccharides (Leeman et al., 1995) and VOCs (Chen et al., 2018a) can also trigger ISR. Some biocontrol agents can lead to ISR in different plant species, while other biocontrol agents show plant species specificity, suggesting specific recognition between microorganisms and receptors on the root surface (Choudhary et al., 2007). An important determinant of biocontrol efficacy is the population density of ISR-triggering microorganisms. For example, ~10^5 CFU of *Pseudomonas defensor* (ex *fluorescens*) WCS374 per g of root are required for significant suppression of Fusarium wilt of radish (Raaijmakers et al., 1995). Another important feature of ISR in plants is that its effects are not only expressed at the site of induction but also in plant parts that are distant from the site of induction (Pieterse et al.,

2014). For example, root-colonizing *Pseudomonas simiae* (ex *fluorescens*) WCS417r induced resistance in carnation, with phytoalexin accumulation in stems, and protected shoots from *F. oxysporum* (Van Peer et al., 1991). Priming of barley heads with *P. fluorescens* MKB158 led to changes in the levels of 1203 transcripts (including some involved in host defense responses), upon inoculation with pathogenic *F. culmorum* (Petti et al., 2010).

Table 3. Biocontrol agents, plant-*Fusarium* systems and ISR mechanisms.

Biocontrol agent	Plant	Pathogen	Mechanism	References
Bacillus amyloliquefaciens	Tomato	*F. oxysporum*	Induction of genes coding for lipoxygenase or pathogenesis-related (PR) proteins, i.e., acidic protein PR-1 and PR-3 chitinases	Aydi Ben Abdallah et al., 2017
Bacillus thuringiensis	Tomato	*F. oxysporum*	Increase in polyphenol oxidase, phenyl ammonia lyase and peroxidase in plant	Akram et al., 2013
Bacillus megaterium	Tomato	*F. oxysporum*	Induction of chitinase, β-1,3-glucanase, peroxidase and polyphenol oxidase activities in plant	Amer et al., 2014
Bacillus subtilis	Tomato	*F. oxysporum*	Increased activities of phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase enzymes in plant	Akram et al., 2015
Bacillus velezensis	Wheat	*F. graminearum*	Production of surfactin and VOCs, activating defense response in plant	Chen et al., 2018
Bacillus subtilis and *Pseudomonas protegens* (in combination and alone)	Chilli	*F. solani*	Increased activities of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, β-1,3-glucanase, chitinase enzymes and phenol compounds involved in the synthesis of phytoalexins	Sundaramoorthy et al., 2012
Bacillus sp., *Brevibacillus brevis* and *Mesorhizobium ciceri* (in combination)	Chickpea	*F. oxysporum*	Increase in peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, phenols and total proteins in plants	Kumari and Khanna, 2019
Brevibacillus parabrevis	Cumin	*F. oxysporum*	Increase in peroxidase and polyphenol oxidase in plants	Abo-Elyousr et al., 2022
Burkholderia gladioli	Saffron	*F. oxysporum*	Increased levels of endogenous jasmonic acid (JA) and expression of JA-regulated and plant defense genes	Ahmad et al., 2022
Pseudomonas aeruginosa	Tomato	*F. oxysporum*	Bacterial production of 3-hydroxy-5-methoxy benzene methanol	Fatima and Anjum, 2017
Pseudomonas simiae	Tomato	*F. oxysporum*	Bacterial production of lipopolysaccharides	Duijff et al., 1997
Pseudomonas defensor	Radish	*F. oxysporum*	Bacterial production of lipopolysaccharides	Leeman et al., 1995
Paenibacillus polymyxa	Cucumber	*F. oxysporum*	Bacterial production of fusaricidin, which induces ISR via salicylic acid	Li and Chen, 2019

P. fluorescens	Barley	*F. culmorum*	Changed transcript levels of lipid transfer proteins and protease inhibitors	Petti et al., 2010
Streptomyces enissoca esilis	Tomato	*F. oxysporum*	Increased catalase activity in plant	Abbasi et al., 2019
Streptomyces rochei	Tomato	*F. oxysporum*	Increased catalase and peroxidase activity in plant	Abbasi et al., 2019
Streptomyces bikiniensis	Cucumber	*F. oxysporum*	Increased activities of peroxidase, phenylalanine ammonia-lyase, and β-1,3-glucanase in plant	Zhao et al., 2012
Trichoderma gamsii	Maize	*F. verticillioides*	Enhanced transcript levels of ISR marker genes	Galletti et al., 2020
Trichoderma longibrachiatum	Onion	*F. oxysporum*	Accumulation of 25 stress-response metabolites	Abdelrahman et al., 2016
Non-pathogenic *Fusarium oxysporum*	Tomato	*F. oxysporum*	Increased activities of chitinase, β-1,3-glucanase and β-1,4-glucosidase	Fuchs et al., 1997

B. amyloliquefaciens subsp. *plantarum* strain SV65 was assessed on tomato plants infected or not with FOL. The expression of genes coding for lipoxygenase or pathogenesis-related (PR) proteins, i.e., acidic protein PR-1 and PR-3 chitinases, was induced by *B. amyloliquefaciens* subsp. *plantarum* SV65 in both FOL-inoculated and uninoculated plants, suggesting its ability to induce ISR (Aydi Ben Abdallah et al., 2017). Inoculation of chilli plants with *B. subtilis* EPCO16 and EPC5 and *P. protegens* Pf1, separately or in combination, induced ISR, with enhanced phytoalexin activities, and protected plants against *F. solani* (Sundaramoorthy et al., 2012). Inoculation of chickpea plants with a combination of *Bacillus* sp., *Brevibacillus brevis* and *Mesorhizobium ciceri* led to the accumulation of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenols in plants as well as resistance to *F. oxysporum* (Kumari and Khanna, 2019). *P. polymyxa* WLY78 controls Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cucumerinum*, through the production of fusaricidin, which can induce ISR in cucumber via the salicylic acid pathway (Li and Chen, 2019). Tomato showed increased catalase and peroxidase activities when treated with *Streptomyces* sp. IC10 and Y28, or with Y28 alone, respectively, outlining a strain-specific ISR in tomato against Fusarium wilt mediated by FOL (Abbasi et al., 2019). *Streptomyces bikiniensis* increased the activities of peroxidase, phenylalanine ammonia-lyase and β-1,3-glucanase in cucumber leaves (Zhao et al., 2012). In the case of *F. graminearum*, it was shown that *B. velezensis* LM2303 induces systemic resistance in wheat by surfactin and VOCs production (Chen et al., 2018a). Non-pathogenic *F. oxysporum* Fo47 can triger induced resistance to FOL and protect tomato from Fusarium wilt (Fuchs et al., 1999). *T. gamsii* IMO5 increased transcript levels of ISR-marker genes *ZmLOX10*, *ZmAOS* and *ZmHPL* in maize leaves, thereby protecting the plant from *F. verticillioides* (Galletti et al., 2020). In soils suppressive to Fusarium wilt of banana, in China, it was shown that *Pseudomonas* may be a key taxa involved in this suppression, as it stimulated immunity in banana, by increasing the levels of jasmonate and salicylic acid, and activities of polyphenol oxidase (Lv et al., 2023).

2.3.5. Inhibition and detoxification of mycotoxins

Biocontrol research often focuses on pathogen inhibition, and effects on mycotoxin synthesis or detoxification are often neglected (Pellan et al., 2020). It can be expected that *Fusarium* inhibition will diminish mycotoxin synthesis, but one comprehensive study found that *B. amyloliquefaciens*

FZB42 inhibited growth of *F. graminearum,* while simultaneously stimulating biosynthesis of DON toxin (Gu et al., 2017). Conversely, DON production of *F. graminearum* (on wheat kernels) was reduced by more than 80% with *B. amyloliquefaciens* WPS4-1 and WPP9 (Shi et al., 2014), and *P. polymyxa* W1-14-3 and C1-8-b (He et al., 2009), whereas *Pseudomonas* strains MKB158 and MKB249 significantly reduced DON production in *F. culmorum*-infected wheat seeds (Khan and Doohan, 2009). *Pseudomonas* sp. MKB158 lowered expression of the gene coding for trichodiene synthase (an enzyme involved in the production of trichothecene mycotoxins in *Fusarium*) by 33%, in wheat treated with *F. culmorum* (Khan et al., 2006). DON production in both *F. graminearum* and *F. verticillioides* was also inhibited by the fungus *T. asperellum* TV1 and the oomycete *Pythium oligandrum* M1/ATCC (Pellan et al., 2020). Other mycotoxins may be targeted, as *Trichoderma harzianum* Q710613, *T. atroviride* Q710251 and *T. asperellum* Q710682 decreased ZEA production in a dual-culture assay with *F. graminearum* (Tian et al., 2018), and *Streptomyces* sp. XY006 lowered the synthesis of fusaric acid in *F. oxysporum* f. sp. *cubense* (Wang et al., 2023).

2.4. Plant-growth promoting modes of action of beneficial bacteria

Besides conferring plant protection from the phytopathogens, bacteria are able to promote plant uptake of essential nutrients (nitrogen fixation, phosphate solubilization and siderophores production) and to alter plant hormonal status (production of phytohormones), through different indirect modes of action (Glick, 2012). In such a way, PGPR facilitates plant growth, contributes to plant fitness, and indirectly enhances tolerance to abiotic and biotic stressors.

Nitrogen fixation. Nitrogen is indispensable for plant growth and development, and its availability significantly affects the plant yield and quality. However, it is estimated that 78-79% of nitrogen is found in the atmosphere in gaseous form (N_2), which is unavailable for plants that have the ability to use nitrogen in the form of nitrate and ammonia (Franche et al., 2009). Certain bacteria and archaea have the ability to fix atmospheric nitrogen and to reduce it to ammonia, in a process catalyzed by enzyme nitrogenase, whose production is coded by the *nif* gene cluster (Bruto et al., 2014). These bacteria, termed diazotrophs, can be in associative symbioses with woody plants and grasses, in nodule symbioses with legume roots or free-living in soils and water. For over a century, scientists have been fascinated by nitrogen-fixing microbial associations in non-legumes, especially cereals (Mus et al., 2016). *Setaria viridis* and *Setaria italica* have been found to acquire nitrogen through associations with *Azospirillum brasilense* (Okon et al., 1983; Pankievicz et al., 2015). Similar associations were discovered in plants like Kallar grass, rice, maize and wheat (Boddey et al., 1991; Hurek et al., 2002; Iniguez et al., 2004; Deynze et al., 2018). Among the free-living, nitrogen-fixing bacteria, it was shown that *P. protegens* Pf-5 X940 significantly raised nitrogen content in wheat and positively affected its growth under the greenhouse conditions (Fox et al., 2016). Eleven strains of *Azotobacter chroococum* were tested for their impact on wheat, demonstrating that all of them augmented plant N contents and yield (Kızılkaya, 2008), while the nitrogen-fixing *Azospirillum* strains were able to increase N content in wheat plants, even under the conditions of salinity stress (El-Akhdar et al., 2019).

Phosphate solubilization. Phosphorus is limiting nutrient for plants, as it is an essential component of many biological molecules, such as ATP, nucleic acids, enzymes and phospholipids (Schachtman et al., 1998). In the soil, it is present in insoluble mineral and organic form. Plants can take phosphorus only in dissolved form, and since most of the phosphorus in the soil is bound in stable chemical compounds, only a small amount of phosphorus is available to the plant

(Hariprasad and Niranjana, 2009). Plants take phosphorus from the soil in the form of orthophosphate ions: either HPO_4^{-2} or $H_2PO_4^-$, and absorb it through root hairs, thereby incorporating phosphorus into organic matter (Schachtman et al., 1998). A large number of microorganisms have the ability to mineralize organic forms of phosphorus with the help of enzymes acid phosphatases and phytases (Duff et al., 1994). Besides, majority of soil bacteria have the ability to solubilize phosphates from its insoluble mineral forms. These microorganisms produce organic acids of low molecular weight, that attack the phosphate structure and transform phosphorus into a form that can be absorbed by the plant. Research showed that the solubilization of phosphates is a consequence of the decrease in pH due to the production of organic acids, which are the source of H^+ ions responsible for the dissolution of mineral phosphates (Alori et al., 2017). Organic acids that solubilize phosphates are gluconic and 2-ketogluconic acids, whose production is coded by the genes *gcd* and *gad*, respectively (Miller et al., 2010), as well as other acids, such as oxalic, maleic, tartaric, acetic, citric and lactic acid (Alori et al., 2017). Inoculation of wheat plants with two phosphate solubilizing-*Pseudomonas* L3 and P2 (separately and in consortia), capable of producing citric and maleic acid, respectively, contributed to longer shoot and root, as well as to better vigor parameters of inoculated wheat plants, compared to non-inoculated control (Dasila et al., 2023). Phosphate-solubilizing and siderophore-producing *B. subtilis* 1 significantly increased wheat shoot and root length, even under saline conditions (Jabborova et al., 2020), while wheat seed coating with phosphate-solubilizing *Bacillus-Rhizobium* consortia led to improved P uptake, growth parameters and yield (Akhtar et al., 2013). Additionally, it was shown that *Bacillus altitudinis* WR10 produces phosphatases and phytases, and in such a way, it reduces wheat stress in the presence of unavailable phosphates (Yue et al., 2019). *Acinetobacter* sp. WR922 produced gluconic acid and contributed to an increase in P content and dry matter in wheat (Ogut et al., 2010).

Production of siderophores. Besides having a role in competition for iron with different phytopathogens, siderophores promote plant growth by helping plants to acquire iron. Iron is a micronutrient, which is essential for plant growth, and in the soil, it is found in an inaccessible form, Fe^{3+} (Ahmed and Holmström, 2014). Siderophores are small, organic compounds, that are produced by microorganisms in conditions of iron deprivation. In these conditions, microorganisms synthesize siderophores that have a high affinity for Fe^{3+} iron, bind it, and transport it into the cell. In the cell, Fe^{3+} is converted to Fe^{2+} form, which is accessible to the microorganisms (Saha et al., 2015). Microorganisms produce a wide variety of siderophores, which are classified based on the functional group, into the following categories: catecholates, hydroxamates and carboxylates (Ahmed and Holmström, 2014). Besides, there are siderophores, such as pyoverdine (whose production is encoded by the gene *pvdL*; Schalk and Guillon, 2013) and pyochelin (whose production is encoded by the gene cluster *pch*; Reimmann et al., 2001), which contain a combination of main functional groups. This abundance of siderophore types (for example more than 50 different pyoverdine siderophores in the *Pseudomonas* genus, each with different peptide side chain), enables characterization of microorganisms based on the type of siderophores they produce, this characterization being called siderotyping (Fuchs et al., 2001). For example, *P. putida* produced an efficient siderophore complex that significantly affected the wheat uptake of labeled ^{59}Fe (Rasouli-Sadaghiani et al., 2014), while *P. stutzeri* produced desferrioxamine E siderophore that help the increase of Fe in wheat plants (Mahajan et al., 2021). Study on *Bacillus* sp. WR13, a wheat endophyte, showed that it has the ability to activate genes coding for siderophore synthesis under Fe-limiting conditions (Yue et al., 2023), and genomic analysis of *B. subtilis* showed the presence of genes involved in synthesis of several siderophores, such as bacillibactin, enterochelin and mixochelin (Dunyashev et al., 2021).

Production of phytohormones. Phytohormones produced by bacteria include auxins, cytokinins, gibberellins, ethylene, and abscisic acid, to which plants are capable of responding. Various types of auxins have been identified, and it has been demonstrated that they enhance plant growth and development by influencing cell division, elongation, and tissue differentiation (Goswami et al., 2016). One extensively studied auxin is indole-3-acetic acid (IAA). More than 80% of rhizosphere bacteria are capable of producing IAA through different synthesis pathways. Some pathways are dependent on the presence of L-tryptophan, which serves as a precursor for IAA synthesis, while others are independent of this amino acid (Idris et al., 2007). Certain microorganisms, such as *Azospirillum*, can produce IAA through a L-tryptophan-independent pathway (Goswami et al., 2016). The more common L-tryptophan-dependent synthesis can occur through pathways such as the tryptamine pathway, indole-3-acetamide pathway (encoded by *iaaMH* genes; Bellés-Sancho et al., 2022), indole-3-pyruvate decarboxylase pathway (encoded by *ipdC* or *ppdC* gene), tryptophan side-chain oxidase pathway, and the indole-3-acetonitrile pathway (Gruet et al., 2022).

Similarly to auxins, plants respond to exogenous cytokinins by exhibiting enhanced hair root formation, shoot initiation, root development, and cell division (Goswami et al., 2016). Over 30 cytokinins have been documented so far, with adenine-type cytokinins being the most common. These cytokinins have an isoprenoid (as found in zeatin) or an aromatic side chain (as found in kinetin) on the N6 position instead of adenine (Maheshwari, 2013). For example, it was shown that cytokinin-producing *Pseudomonas* G20-18 promoted growth of radish and wheat plants (García de Salamone et al., 2001), while *P. fluorescens* AK1 and *P. aeruginosa* AK2 produced cytokinins in the presence of rice exudates (Karnwal and Kaushik, 2011).

Gibberellins, diterpenoid acids synthesized by higher plants and certain microorganisms (Maheshwari, 2013), also play a role in cell division, elongation, seed germination, root growth promotion, flowering, and fruit setting (Hedden and Phillips, 2000). Production of gibberellins is documented in microbial species belonging to genera *Bacillus*, *Rhizobium*, *Azospirillum* and *Acetobacter* (Goswami et al., 2016).

Ethylene, a volatile phytohormone primarily produced in plants, exhibits increased levels when plants face various environmental stresses such as salinity, drought, flooding, pollutants, pathogens, and pests. In addition to serving as a stress marker, ethylene regulates flower senescence, fruit ripening, and leaf and petal abscission (Etesami et al., 2015). When plants experience stress, ethylene production peaks, initiating the transcription of PR genes and inducing acquired resistance in plants. If the stress persists, ethylene production may affect abscission, chlorosis, and senescence, significantly impacting plant growth (Gamalero and Glick, 2011). In bacteria, ethylene synthesis is encoded by the *efe* gene, and when produced by certain pathogenic bacteria, i.e., *P. syringae*, ethylene acts as a virulence factor (Van Loon et al., 2006; Wang et al., 2010). On the other hand, bacterial ethylene is considered as an elicitor of ISR in plants (Gamalero and Glick, 2011). The precursor of ethylene synthesis is ACC. Certain microorganisms have the ability to produce ACC deaminase (*acdS* gene; Shah et al., 1998), an enzyme which cleaves ACC molecule to α-ketobutyrate and ammonia, thereby inhibiting the production of ethylene in plants. ACC deaminase-producing microorganisms, such as *Pseudomonas*, *Burkholderia*, *Acinetobacter*, *Stenotrophomonas* facilitate plant growth and development under the stressful conditions (Honma and Shimomura, 1978; Ahemad and Kibret, 2014).

Abscisic acid is a phytohormone involved in plant responses to various environmental stresses. Increased levels of abscisic acid have been observed in plants subjected to drought, cold, salt and wounding conditions (Mehrotra et al., 2014). Under such conditions, abscisic acid

modulates physiological processes that regulate plant response to these stresses. It affects stomatal closure, modifies cell wall elasticity, improves water uptake and enhances transpiration efficiency (Cohen et al., 2015).

Many bacteria have the ability to produce multiple phytohormones. For instance, *P. fluorescens* and *Burkholderia caryophylli*, which produce ACC deaminase and IAA, have demonstrated significant enhancement in wheat yield and growth under field conditions (Shaharoona et al., 2007). Similarly, *P. aeruginosa*, *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. cereus* and *Azospirillum brasilense* by producing cytokinins and IAA have led to increased levels of these phytohormones in wheat (Hussain and Hasnain, 2011). *B. subtilis* HG-15 produced IAA, abscisic acid, gibberellins and the ACC deaminase, contributing to enhanced wheat plant growth, even under elevated salinity conditions (Ji et al., 2022). *Chryseobacterium gleum* SUK producing IAA and ACC deaminase positively affected wheat growth parameters, such as weight and shoot and root length (Bhise et al., 2017).

Collectively, these examples highlight the diverse PGP mechanisms employed by different bacterial species to protect plants from phytopathogens and enhance their growth, with species from the *Pseudomonas* genus being particularly notable in this regard.

2.5. The importance of *Pseudomonas* in biological control and plant-growth promotion

The proteobacterial genus *Pseudomonas* encompasses species with versatile metabolism and physiology that inhabit diverse aquatic, terrestrial and biotic environments. *Pseudomonas* species display different lifestyles: some are opportunistic pathogens of humans, insects or plants, some are used in bioremediation, while others function as PGPR by providing phytostimulation and/or phytoprotection (Silby et al., 2011). Since its initial discovery by Migula (1894), numerous new species have been added to this genus, now comprising over 315 validly published species (List of Prokaryotic Names with Standing in Nomenclature; https://lpsn.dsmz.de/genus/pseudomonas, accessed on September 10th, 2023). MultiLocus Sequence Analysis (MLSA) of four housekeeping genes (the 16S rRNA gene *rrs*, *gyrB*, *rpoB* and *rpoD*) and comparisons of Average Nucleotide Identity (ANI) revealed three distinct lineages within the *Pseudomonas* genus, referred to as the *P. aeruginosa*, *P. fluorescens* and *P. pertucinogena* lineages (Peix et al., 2018). However, this classification has shown inconsistencies, as the genus *Pseudomonas* was not monophyletic and included genera such as *Azotobacter* and *Azomonas* (Nikolaidis et al., 2020; Rudra and Gupta, 2021; Saati-Santamaría et al., 2021). In 2021, two articles published one month apart developed a phylogenomic analysis of the genus *Pseudomonas* and proposed a reclassification of the monophyletic lineage of *P. pertucinogena*, which forms a distinct clade from the main *Pseudomonas* clade and consists of halotolerant species, into the genus '*Neopseudomonas*' (Saati-Santamaría et al., 2021) or *Halopseudomonas* (Rudra and Gupta, 2021). They also repositioned the deep-branching species *Pseudomonas hussainii* into a new genus termed '*Parapseudomonas*' (Saati-Santamaría et al., 2021) or *Aptomonas* (Rudra and Gupta, 2021). Another phylogenomic study reclassified the *P. stutzeri nitrogen*-fixing clade and established the new genus *Stutzerimonas* (Lalucat et al., 2022). Alongside the *P. aeruginosa* lineage, the *P. fluorescens* lineage persists in the *Pseudomonas* genus. The *P. fluorescens* lineage (the most diverse and complex) is usually subdivided into subgroups, represented by the species *P. fluorescens*, *P. fragi*, *P. gessardii*, *P. mandelii*, *P. koreensis*, *P. jessenii*, *P. asplenii*, *P. corrugata, P. kielensis, P. protegens* and *P. chlororaphis* (Figure 6; Mulet et al., 2010; Garrido-Sanz et al., 2016; Hesse et al., 2018; Girard et al., 2021). It is known that the *P. fluorescens*

group includes a few phytopathogens (such as *P. corrugata* or *P. mediterranea*; Trantas et al., 2015), as well as various phytobeneficial species.

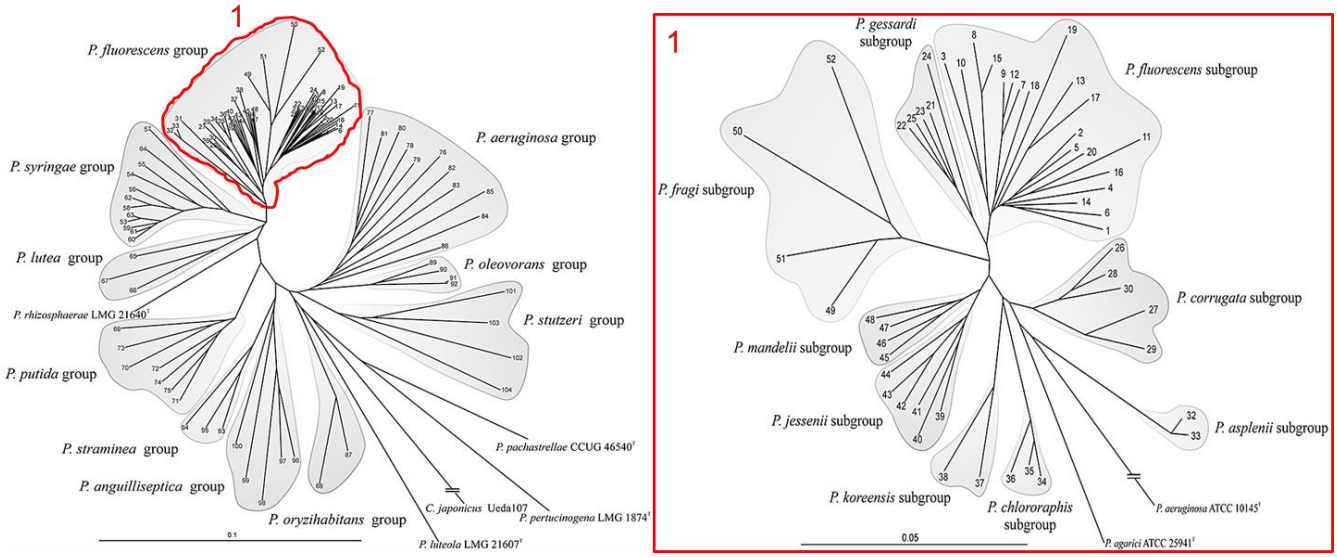

Figure 6. Phylogenetic tree of *Pseudomonas* genus (left) and phylogenetic tree of *Pseudomonas fluorescens* group (right). Trees were constructed based on the concatenated partial sequences of the 16S rRNA gene *rrs*, *gyrB*, *rpoB* and *rpoD* genes. Taken from Mulet et al. (2010).

P. fluorescens group contains species with diverse PGP properties, incuding phytostimulation or phytoprotection, and as such, species of this group have an important role in the rhizosphere (Loper et al., 2012; Sarma et al., 2014; Vacheron et al., 2016). Certain species within this group have the ability to produce a large range of antifungal substances that can inhibit or deactivate the growth of *Fusarium* (Vacheron et al., 2016). These antifungal substances encompass various secondary metabolites with antimicrobial properties, such as pyoluteorin, pyrrolnitrin, DAPG, phenazine, 2-hexyl-5-propyl-alkylresorcinol (HPR) or HCN. Additionally, they produce lytic enzymes such as chitinases, cellulases or proteases (Nowak-Thompson et al., 2003; Loper et al., 2012; Sarma et al., 2014; Vacheron et al., 2016; Kumar et al., 2017), which could directly impede pathogens. *Pseudomonas* may also elicit ISR in plants by producing lipopolysaccharides or flagella, DAPG or siderophores (Bakker et al., 2007). This diverse array of mechanisms has positioned species within the *P. fluorescens* group as prime candidates for biological control since the 1970s (Weller et al., 2007). Indeed, fluorescent *Pseudomonas* strains with biocontrol properties, isolated from soils suppressive to take-all disease of wheat and barley caused by the fungal pathogen *G. graminis* var. *tritici* (Cook and Rovira, 1976) or soils suppressive to *T. basicola*-mediated black root of tobacco (Stutz et al., 1986), effectively protected plants from disease (Almario et al., 2014). In soils suppressive to *F. oxysporum* in Salinas Valley, it was shown that the suppressiveness is attributed to the presence of siderophore-producing, fluorescent *Pseudomonas*, which are more competitive and exhibit faster iron complexation compared to the pathogen (Kloepper et al., 1980; Sneh et al., 1984). In China, *Pseudomonas* present in soils suppressive to *F. oxysporum* induced ISR in banana, by increasing jasmonate and salicylic acid levels, and by enhancing the activity of polyphenol oxidase (Lv et al., 2023). In addition to their phytoprotective role, species of the *P. fluorescens* group have the ability to modulate plant growth by producing phytohormones (Vacheron et al., 2016), by

solubilizing phosphates (Meyer et al., 2010), by fixing nitrogen (Fox et al., 2016), by denitrification (Almeida et al., 1995) and by producing ACC deaminase (Glick et al., 1998; Prigent-Combaret et al., 2008). Consequently, fluorescent *Pseudomonas* are among the most extensively studied PGPRs, capable of promoting plant health through both direct and indirect mechanisms (David et al., 2018). Genome analysis is a valuable tool for understanding different modes of action of these bacteria as it enables the characterization of plant growth-promoting functional traits and bacterial identification (Van Elsas et al., 2008). It was also shown that certain *Pseudomonas* may posses protein secretion systems, such as type III (T3SS), type IV (T4SS) and type VI (T6SS) secretion system, located on the bacterial cell membranes, with purpose of secreting different compounds (Loper et al., 2012). T3SS is found in many Gram-negative species, including certain non-pathogenic *Pseudomonas*, and it may alter plant immunity (Mavrodi et al., 2011) and enhance phytoprotective properties of these bacteria (Rezzonico et al., 2005; Marchi et al., 2013). T4SS is present in many bacterial species, and it was shown that in *P. putida*, it acts as a defense mechanism and protects tomato plants from pathogenic *Ralstonia solanacearum* (Purtschert-Montenegro et al., 2022). T6SS, also found in *P. putida*, mediates inter-bacterial competition, secretes toxic metabolites against phytopathogens and protects *Nicotiana benthamiana* plant from pathogen *Xanthomonas campestris* (Bernal et al., 2017). Considering all of this, research on *Pseudomonas* species, their genomic potential and their modes of action in soils suppressive to *F. graminearum* is of high importance, as these microbes may provide insights into the functioning of these soils and offer potential solutions for combating mycotoxicogenic *F. graminearum*.

3. OBJECTIVES

Crop plants are exposed to a wide range of soil-borne phytopathogens, particularly oomycetes and fungi, which are difficult to control with conventional strategies as they are often inefficient. However, certain microorganisms present in soil may inhibit these pathogens directly, through competition or antagonism, or they can indirectly stimulate other plant-associated microorganisms or induce plant immune responses, providing soil suppressivess to fungal disease(s). Fungistasis is a form of soil suppressiveness that is taking place in the absence of the plant, explained as the soil's ability to inhibit the germination or hyphae growth, and it is a result of competition or antagonism of the entire microbial community present in the soil. Suppressive soils represent the best example of natural microbe-based plant defense, but despite the fact that suppressive soils have been known for more than 70 years, they have not been discovered at many sites, and the microbial basis of suppressiveness remains poorly understood.

Taking all of this into account, the general objective of this project was to gain a better understanding of fungistasis and suppressiveness phenomena, and to assess usefulness of suppressive and fungistatic soils as sources of bacteria with biocontrol potential. To this end, we focused on mycotoxicogenic pathogen *F. graminearum*, as soils suppressive to diseases caused by different *Fusarium* species have been documented in different geographic regions (but not in Serbia), and because this pathogen can be influenced by fungistasis.

In this context, the first objective was to identify soils fungistatic and suppressive to *F. graminearum*, as well as to investigate the relation between manure amendments and the occurrence of fungistasis/suppressiveness.

The second objective aimed to assess the potential of *F. graminearum* fungistatic soils as a source of biocontrol agents. This involved isolation of bacteria of diverse taxonomy, their characterization based on genomic and functional traits, and assessment of their wheat phytoprotective capacity against *F. graminearum*.

The third objective of this work was to identify the genomic and functional particularities of *Pseudomonas* bacteria in suppressive vs. non-suppressive soils. This was motivated by the fact that *Pseudomonas* may contribute to plant protection against *Fusarium* diseases and play a role in soil suppressiveness to these diseases, although biocontrol *Pseudomonas* have also been documented in non-suppressive soils. To achieve this comparison, the diversity of fluorescent *Pseudomonas* in the rhizosphere of wheat grown in suppressive and non-suppressive soils was analyzed using a metabarcoding approach, targeting the *rpoD* gene of the *P. fluorescens* group. Subsequently, *Pseudomonas* representatives were isolated from the rhizospheres of wheat plants grown in these soils and characterized based on genomic and functional traits.

4. MATERIAL AND METHODS

4.1. Soil sampling

A total of 26 agricultural fields were selected and sampled in Serbia, at locations near Sombor (SO), Novi Karlovci (NK), Valjevo (VA), Mionica (MI) and Čačak (CA) (Table 4). At each location, wheat was grown in a crop rotation and fields with or without manure added regularly were sampled. For each location, farmers filled in a questionnaire about the recent cropping history, fertilizers and pesticides application, management of postharvest residues and the observed presence of wheat fusariosis. During the sampling, the first few cm of the top soil were removed, and samples were taken from 5-20 cm depth. Roots, stones and animals were manually removed and all the soils were sieved (0.5 cm). After the sampling, water content was measured by drying at 105°C for 24h. These soils were used in the experiments described in sections 4.2., 4.3. and 4.4. First sampling (for experiment described in section 4.2.) was performed in autumn 2020, and the second sampling (for experiments described in sections 4.3. and 4.4.) was performed in spring 2021.

Additionally, soil located in La Côte-Saint-André (LCSA; Isère, France) (Table 4) was sampled in spring 2022, according to the protocol described above, and used for the experiment within section 4.10.

Table 4. Soil samples - locations, sample ID and its GPS coordinates.

Location	Sample ID	GPS coordinates	
Sombor	SO1	45.758696 N	19.1840320 E
	SO2	45.746168 N	19.159358 E
	SO3	45.750012 N	19.170019 E
	SO4	45.750839 N	19.172977 E
Novi Karlovci	NK1	45.060182 N	20.215013 E
	NK2	45.060066 N	20.215213 E
	NK3	45.088806 N	20.102067 E
	NK4	45.088011 N	20.099312 E
Valjevo	VA1	44.33050 N	19.968102 E
	VA2	44.330491 N	19.966663 E
	VA3	44.330466 N	19.969106 E
	VA4	44.330110 N	19.968102 E
	VA5	44.351892 N	19.981415 E
	VA6	44.351155 N	19.978144 E
	VA7	44.355395 N	19.977465 E
	VA8	44.355012 N	19.977650 E
Mionica	MI2	44.24611 N	20.10431 E
	MI3	44.24540 N	20.10350 E
	MI4	44.24745 N	20.10012 E
	MI5	44.24759 N	20.09931 E
Čačak	CA1	43.89897 N	20.54435 E
	CA2	43.89910 N	20.54450 E
	CA3	43.89905 N	20.54312 E
	CA4	43.89930 N	20.54315 E
	CA5	43.8867833 N	20.5462167 E
	CA6	43.8878667 N	20.5475167 E
La Côte-Saint-André	LCSA	45.37861 N	5.26722 E

4.2. Soil fungistasis to *Fusarium graminearum*

The aim was to evaluate soils' fungistasis towards *F. graminearum*, i.e., fungal growth/survival in different soils. For this purpose, soils were sampled as described in section 4.1., and fungistasis was examined for each of these soils, previously autoclaved and non-autoclaved, in order to assess the impact of the biotic component of these soils on *F. graminearum* survival/growth.

4.2.1. *Fusarium graminearum* Fg1 strain and inoculum preparation

The highly virulent and toxin-producing isolate *Fusarium graminearum* MDC_Fg1 (hereafter termed *F. graminearum* Fg1) used during the experiments was provided by Dr. Thierry Langin (GDEC Joint Research Unit, INRA Center Auvergne-Rhone-Alpes, Clermont-Ferrand, France). This strain was isolated from naturally infected cereal grains in the North of France (Alouane et al., 2018). *F. graminearum* Fg1 was grown on PDA (Potato Dextrose Agar, Condalab, Madrid, Spain) at 20-22°C for eight days when actively growing cultures were needed.

F. graminearum Fg1 inoculum was prepared according to a protocol adapted from Legrand et al. (2019). In brief, 300g of maize kernels was soaked in 750 ml of tap water in a 2-liter Erlenmeyer flask and left for 72h at room temperature. Afterwards, this mixture of water and kernels was grounded into homogenous media (Ø1-2 mm), poured into a 2-liter Erlenmeyer flask and autoclaved for 20 min at 121°C for two consecutive days. After autoclaving, the prepared maize media was left to cool down, and it was inoculated with disks (Ø7 mm) from the edges of eight days-old cultures of *F. graminearum* Fg1 grown on PDA plates. Flasks were kept at room temperature for 10 days, and vigorously shaken every other day to ensure even colonisation of the ground maize by *F. graminearum* Fg1 mycelium.

4.2.2. *Fusarium graminearum* Fg1 genomic DNA extraction

For *F. graminearum* Fg1 DNA extraction, 8-days old fungal mycelium grown on PDA was scraped, transferred to a 1.5 ml Eppendorf tube and lyophilized (for 24h, -50°C, 1.25 mbar; freeze-dryer, Lyophilizator, Alpha 1-4LSC, Christ, Germany). Lyophilized mycelium was ground, and 0.5 ml of the extraction buffer (5 M NaCl, 10 ml; 1 M Tris-HCl (pH=7.5), 1 ml; 0.5 M Na_2EDTA (pH=8), 2 ml; 10% SDS, 10 ml and H_20, 100 ml), previously heated to 65°C, was added and incubated for 10 min at room temperature. After adding 0.5 ml of phenol (pH=8) and 0.5 ml of SEVAG (96 ml of chloroform and 4 ml of isoamyl alcohol), the content was centrifuged (Eppendorf Centrifuge Minispin plus, Hamburg, Germany) at 14500x*g* for 20 min. Supernatant was transferred to a clean 1.5 ml centrifuge tube, one volume of SEVAG was added, the tube was shaken a few times by inversion and centrifuged again at 14500x*g* for 10 min. Supernatant was transferred to a clean 1.5 ml centrifuge tube, one volume of cold isopropanol was added, the tube was shaken a few times by inversion, incubated at -20°C for 10 min and centrifuged at 14500x*g* for 10 min. Supernatant was discarded, the DNA pellet was rinsed by adding 0.5 ml of 70% ethanol and centrifuged at 14500x*g* for 5 min. Supernatant above the DNA was discarded, and the DNA was left for 10 min at room temperature, before mixing it with 100 µl of ultra-pure H_2O. DNA concentration was determined using a UV Spectrophotometer (NanoPhotometer NP80, Implen, Munich, Germany) and the extracted DNA stored at 4°C for further use.

4.2.3. Evaluation of soil fungistasis to *Fusarium graminearum* Fg1

Prior to inoculation, four 1-g autoclaved samples and four 1-g non-autoclaved samples of each soil, as well as four 1-ml samples of *F. graminearum* Fg1 inoculum, were collected and stored at -20°C before quantifying *F. graminearum* Fg1. The experiment was done in 20-ml vials containing 15 g soil, which was autoclaved (for 20 min at 121°C on two consecutive days) or not, and then inoculated (600 µl of mycelia inoculum) or not (600 µl of water), giving for each of the 26 soils (i) 4 inoculated, autoclaved vials, (ii) 4 inoculated, non-autoclaved vials, and (ii) 4 non-inoculated, non-autoclaved vials, i.e., 26 × (4 + 4 + 4) = 312 vials. The vials were arranged following a randomized block design and incubated in the dark at 60% air humidity and 20°C. Every three days, vials were weighted to estimate water loss, and the corresponding amount was added back. After 15 days, all soil samples were lyophilized (Lyophilizator, Alpha 1-4LSC, Christ, Germany) for 48 h, 1 g soil was sampled from each vial and stored at -20°C until DNA extraction.

Total DNA was extracted from 0.5 g soil for each of the 520 samples (208 samples before inoculation and 312 samples at 15 days) and from 1 ml of each Fg1 inoculum sample (4 samples), using FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch-Graffenstaden, France), according to manufacturer's instructions. *F. graminearum* Fg1 DNA was quantified by qPCR using a CFX-96TM Real-Time PCR System (Bio-Rad, Hercules, CA, USA). qPCR was performed in a total mix of 20 µl, containing 10 µl of SensiFAST SYBR No-ROX master mix (Bioline, Meridian Bioscience, Cincinnati, OH, USA), 1 µl of primers Fg16N-F (5'-ACAGATGACAAGATTCAGGCACA-3') and Fg16N-R (5'-TTCTTTGACATCTGTTCAACCCA-3') (Nicholson et al., 1998) at a final concentration of 20 µM, 6 µl of DNAse-free water and 2 µl of DNA sample. The qPCR cycle program consisted of 2 min denaturation at 98°C, and 40 cycles of denaturation at 98°C for 3 s and annealing/extension at 60°C for 3 s. All samples were run in triplicate, and negative controls were included to each run. Melting curve and Melting temperature (Tm) were determined using the Tm Calling Analysis module of LightCycler Software (v.1.5; Roche Applied Science, Meylan, France), and Cycle threshold (Ct) of each sample was determined with the second derivative maximum method in the LightCycler Software (v.1.5; Roche Applied Science). A melting curve was generated at the end of each qPCR run with a temperature gradient of 0.5 °C.s^{-1} from 60°C to 95°C (melting temperature of *F. graminearum* Fg1 amplicons was at 80°C). Only the amplicons with Tm ~ 80°C were considered as positive, while for all the amplicons with Tm different from 80°C, concentration of *F. graminearum* Fg1 in the sample was replaced by the quantification limit of 4.95×10^5 gene copies.g^{-1} dry soil. The standard curve was generated by plotting the mean Ct value of the three replicates (per DNA concentration) against DNA concentration. Amplification efficiency (E), calculated as $E = 10^{(1/slope)} - 1$, and the Mean Squared Error (MSE) of the standard curve were determined. Quantification of amplicons was achieved using a standard curve generated from serial dilutions (in three replicates) of previously extracted *F. graminearum* Fg1 genomic DNA ranging from 1.77×10^{-8} g.µl^{-1} to 1.77×10^{-11} g.µl^{-1}. Results obtained in g.µl^{-1} were transformed into numbers of copies.g^{-1} soil using the formula [DNA (g) × Avogadro's number (molecules.mol^{-1})] / [number of DNA matrix bp in amplified fragments × 660 (g.mol^{-1})], based on an average of 660 g.mol^{-1} per base pair. They were normalized to the total DNA quantity extracted from 0.5 g of soil and expressed into a number of copies.g^{-1} dry soil as previously done (Bouffaud et al., 2016). The amount in the Fg1 inoculum was calculated for 1 ml (same calculation as for 1 g of soil), extrapolated to the 600 µl used to inoculate 15 g of soil, and expressed per g of soil. This amount was subtracted from the DNA quantity found in each sample of 1 g of soil. All results were $\log_{10}$-transformed for subsequent analysis. Mean values and

standard deviation were calculated. The fungistasis level was computed according to a formula adapted from Legrand et al. (2019):

$$\Delta day15 = \log10(\text{FgDNA in soil at day 15 after inoculation}) - \log10(\text{FgDNA in the inoculum})$$

4.3. *In planta* suppressiveness assay

Soils from location near Mionica, Serbia i.e., MI2, MI3, MI4 and MI5 (Table 4), were chosen for a greenhouse, *in planta* suppressiveness assay with *F. graminearum* Fg1. In contrast to soils' fungistasis, which is a result of direct mechanisms of control of the fungal pathogen's growth/survival by the soil microbiota, soils' suppressiveness may also be a result of various indirect mechanisms, e.g., induced systemic resistance, phytohormone production, etc., when a plant is present in the system examined. Therefore, the aim of the section 4.3. was to assess soils' suppressiveness to pathogen in the presence of the wheat plant.

4.3.1. Preparation of *Fusarium graminearum* Fg1 spore suspension

F. graminearum Fg1 spore suspension was prepared by growing the fungus in Mung Bean Broth (MBB) (Evans et al., 2000). MBB was prepared by adding 40g of organic mung bean seeds in 1l of boiling water and leaving to infuse and cool down for 10 min. After that, beans were discarded and 50 ml of the resulting media was poured into 250 ml Erlenmeyer flasks and autoclaved for 20 min at 121°C. After completely cooling down, prepared MBB was inoculated from an 8-days old *F. graminearum* Fg1 PDA plate (10 patches x Ø7 mm culture in each 250 ml Erlenmeyer flask) and incubated for six days at 22°C with 180 rpm agitation (Incubator Shaker Series I26, New Brunswick Scientific Co., Inc., Edison, New Jersey, USA). After six days of incubation, a volume of preculture was taken and diluted to one tenth in fresh MBB medium and incubated under the same conditions for 10 days. Obtained culture was shaken, filtered using sterile Miracloth to discard mycelium and centrifuged at 4700x*g* for 10 min at room temperature (Avanti J-E Series, Beckman Coulter, Fullerton, USA). Supernatant was discarded and the resulting pellet was washed twice with sterile water. Titration of spores in the suspension was estimated using a Thoma counting chamber (Marienfeld, Germany).

4.3.2. Suppressiveness assay

Suppressiveness assay with *F. graminearum* Fg1 was performed in the plant growth chamber (FitoClima, 10.000 EH, ARALAB, Rio de Mouro, Portugal), with the following conditions: 16h day at 20°C/8 h dark at 18°C and relative humidity 80%. Seeds of winter wheat (*Triticum aestivum* L.) variety Récital were provided by Dr. Thierry Langin (GDEC Joint Research Unit, INRA Center Auvergne-Rhone-Alpes, Clermont-Ferrand, France). For each MI soil, sampled as described in section 4.1., 100 wheat seeds were distributed in 20 pots (12 x 10 x 10 cm) filled with 250 g of soil mixed with sterile siliceous sand (granulometry 0.6-1.6 mm, Gedimat, Dagneux, France; autoclaved twice, at 24 h interval) in a 50:50 ratio. Half of the seeds were inoculated with 100 µL of the prepared *F. graminearum* Fg1 spore suspension (10^6 spores per seed), while half were inoculated with 100 µL of water. The experiment followed a randomized block design with 10 blocks (n = 10). Plants were watered every three days maintaining soil close to 21% w/w water content. After 14 days, the number of germinated seeds was recorded, and (i) the number of

plants alive, (ii) shoot length (cm), (iii) dry shoot biomass (mg), and (iv) dry shoot density (i.e., shoot length divided by dry shoot biomass; mg/cm) were measured at 28 days.

4.4. Analysis of prokaryotic and fungal rhizosphere diversity through metabarcoding approach

4.4.1. Separation of rhizosphere and DNA extraction

Both *F. graminearum* Fg1-inoculated and non-inoculated wheat plants grown for 28 days during the experiment from section 4.3.2. were used to sample the root system from six blocks, with one plant per pot. Loosely-adhering soil was discarded by shaking. Roots and tightly-adhering soils were frozen in liquid nitrogen, lyophilized for 48 h and then stored at -20°C. Root-adhering soil was mechanically separated (using sterile tweezers) and 0.5 g of soil was used for DNA extraction with the FastDNA SPIN kit for Soil and the FastPrep instrument (MP Biomedicals), following manufacturer's instructions. DNA was eluted in 80 µl DNase-free water and quantified using Qubit dsDNA High sensitivity Assay Kit with an Invitrogen Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA quality was assessed using a UV spectrophotometer (NanoPhotometer NP80, Implen, Munich, Germany).

4.4.2. 16S rRNA gene and ITS sequencing from rhizospheric DNA

A PCR reaction amplifying the V3-V4 region of the 16S rRNA gene using primers Uni341F (5′ CCTAYGGGRBGCASCAG 3′) and Uni806R (5′ GGACTACHVGGGTWTCTAAT 3′) (Yu et al., 2005; Caporaso et al., 2011; Sundberg et al., 2013) was performed in a GeneTouch Plus Thermal Cycler (Biozym Scientific, Hessisch Oldendorf, Germany). The PCR reaction contained 14.6 µl of molecular-grade water, 2.5 µl of 10 × standard reaction buffer (New England BioLabs, Ipswich, MA, USA), 1 µl of 25 mM MgCl2, 1.25 µl of BSA (Bovine Serum Albumin; 2 $mg.ml^{-1}$; New England BioLabs), 2.5 µl of 2 mM dNTPs, 1 µl of each primer (0.4 µM), 0.125 µl of Hot Start DNA polymerase (5 $U.\mu l^{-1}$; New England BioLabs), and 1 µl of DNA template (5-10 $ng.\mu l^{-1}$) in a total mix of 25 µl. The PCR reaction included an initial activation step at 94°C for 2 min, followed by 30 cycles of 94°C for 20 s, 56°C for 20 s, 72°C for 40 s, and a final extension step at 72°C for 5 min. The same PCR process and conditions were used to generate libraries from the 16S rRNA gene amplicons, using primers Uni341F/Uni806R with Illumina adaptors (Nextera XT Index Kit, Illumina, San Diego, CA, USA) at Novogene (Cambridge, UK), using Illumina MiSeq v.2 (2 × 250 bp) chemistry, following the manufacturer's instructions (Illumina).

The fungal ITS2 region was amplified using the primers fITS7 (5' GTGARTCATCGAATCTTTG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Gardes and Bruns, 1993; Ihrmark et al., 2012). Primers were equipped with Illumina adaptors (Nextera XT Index Kit, Illumina). To obtain high-fidelity amplification, PCR was performed using Kapa Hifi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA). The PCR was done in triplicate in a S1000 Thermal Cycler (Bio-Rad), with an initial denaturation at 95°C for 5 min, followed by 33 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 75 s and a final elongation step at 72°C for 10 min. PCR products were purified using AMPure XP beads. To assign the sequences to the respective samples, an index PCR was performed using the Illumina Nextera XT Index Kit and Kapa Hifi HotStart ReadyMix (KAPA Biosystems), according to the manufacturer's instructions. PCR products were purified again with AMPure XP beads and quantified with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) following the manufacturer's instructions. For sequencing, samples

were pooled, and the pools were checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Paired-end Illumina MiSeq sequencing (2 × 300 bp) was performed at the Department of Soil Ecology, UFZ-Helmholtz Centre for Environmental Research in Halle (Saale, Germany).

4.4.3. Sequence data processing

Amplicon sequencing datasets from 16S rRNA gene and ITS were handled independently. Sequences from the 16S rRNA gene dataset were processed and classified using the R package DADA2 (Divisive Amplicon Denoising Algorithm) v.1.12.1 pipeline (Callahan et al., 2016). Using the "FilterAndTrimmed" function, quality filtering and trimming stages were executed. Reads shorter than 100 bp were removed, allowing two errors per read. ITS sequences were processed using dadasnake v.10 (Weißbecker et al., 2020; https://github.com/a-h-b/dadasnake), with the DADA2 package in R (v.3.6.1; Callahan et al., 2016). Only reads with the expected amplification primers were kept, and primer sequences were cut using cutadapt v.1.18 (Martin, 2011). The amplicon reads were truncated to a minimum base quality of 7, with a minimum length of 70 nucleotides for the forward and reverse reads. For both datasets, read pairs were merged with zero mismatches, and exact sequence variants were determined as ASVs (Amplicon Sequence Variants). Chimeric reads were removed using the DADA2 "consensus" algorithm. For the 16S rRNA gene dataset, the ASVs were assigned taxonomically using the SILVA database v.138 (Quast et al., 2013), while the UNITE database v.9 was used to assign the ITS2 gene amplicon sequences taxonomically using the mothur implementation of the Bayesian Classifier (Schloss et al., 2009). During this process, ASVs identified as chloroplasts, mitochondria, or eukaryotes in the 16S rRNA gene sequences were excluded from the analysis. The phyla nomenclature was maintained as suggested by the Silva database v.138 (Quast et al., 2013). The 16S rRNA gene primers have been designed to target both the archaeal and bacterial domains; hereafter, this subset of the microbiota is reffered to as the prokaryotic community. For ITS, all ASVs assigned to fungi were kept. Prokaryotic and fungal taxa were identified at the genus level when possible, otherwise at the family or the order level. For 16S rRNA gene and ITS, the raw amplicon data were deposited into the NCBI Sequence Read Archive (SRA).

4.5. Formation of indigenous biocontrol bacterial collection

The goal of this part of the experiment was to assess the usefulness of fungistatic and non-fungistatic soils as sources of biocontrol agents against *F. graminearum* Fg1. Aiming to isolate rhizosphere bacteria with potential biocontrol activity against *F. graminearum* Fg1, rhizosphere extract of MI and CA soils was plated on different media, grown bacteria were picked and purified and *in vitro* confrontation test with *F. graminearum* Fg1 was performed.

4.5.1. Preparation of the rhizosphere soil extract

Wheat plants (*Triticum aestivum* L.), variety Récital, were grown in soils from a site near Mionica (MI), Serbia, i.e., MI2, MI3, MI4 and MI5, and near Čačak (CA), Serbia, i.e., CA1, CA2 and CA3 (Table 4), for 28 days. Wheat plants were harvested, their roots were separated, shaken vigorously and, afterwards, rhizosphere was sampled using an adapted protocol from Bulgarelli et al. (2012), and rhizosphere extract was used for bacterial isolation. In brief, for each soil, wheat root systems with adhering soil were put in 50 mL of phosphate buffered saline (NaCl, 8 g; KCl, 0.2 g; KH_2PO_4, 0.24 g; Na_2HPO_4, 1.42 g; H_2O, 1000 mL) and shaken for one hour at 160 rpm

(Innova 42R, New Brunswick Scientific, Edison, New Jersey, USA). The roots were discarded, the suspension was centrifuged at 4000x*g* for 20 min (Avanti J-E Series, Beckman Coulter, Fullerton, USA), after which the supernatant was discarded. The resulting pellet was mixed with 20 mL of 0.8% NaCl, vortexed, and the obtained suspension represented the rhizosphere soil extract.

4.5.2. Isolation of indigenous rhizosphere bacteria on different media

Isolation of diverse bacterial isolates was done by plating serially diluted rhizosphere extract of wheat plants grown in soils MI and CA (prepared as described in section 4.5.1.) on different general media, i.e., Nutrient Agar (NA; Carl Roth, Karlsruhe, Germany) and Tryptone Soya Agar (TSA; Carl Roth, Karlsruhe, Germany), as well as on selective media: Citrimide Agar (Merck, Darmstadt, Germany) and King's B agar (Condalab, Madrid, Spain) for *Pseudomonas*, Fiodorov agar (Anderson, 1958) for *Azotobacter*, and Starch Ammonia Agar (SAA; starch, 10 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4$ x $7H_2O$, 1 g; NaCl, 1 g; KNO_3, 1 g; $CaCO_3$, 3 g; agar, 20 g; H_2O up to 1L) for *Actinomycetes.* Additionally, one part of the rhizosphere extract was pasteurized at 80°C for 10 min and plated on NA, aiming to isolate representatives from the genus *Bacillus*. All plates were incubated at 28°C in the dark until the bacterial growth had occurred, single colonies were picked and purified until pure cultures were obtained. Bacteria were checked for purity and differentiated as Gram-positive or Gram-negative, using the Gram staining technique, and stored at -80°C in 25% glycerol.

4.5.3. Confrontation test of indigenous rhizosphere bacteria and *Fusarium graminearum* Fg1

Confrontation test with isolated rhizosphere bacteria and *F. graminearum* Fg1 was performed on PDA plates. PDA plates were inoculated with discs (Ø7 mm) taken from the edges of 8-days old *F. graminearum* Fg1 colony and a streak of each bacterium from the bacterial collection was made 3 cm apart from the pathogen. In the case of *Actinomycetes*, bacteria were firstly inoculated on PDA plates, and after 5 days, fungal discs were added 3 cm apart from the bacteria. Control plates were inoculated with *F. graminearum* Fg1 discs only. Each confrontation was performed in triplicates. Plates were incubated in the dark at 22°C for seven days, when observed changes in pathogen growth were noted, and for 14 days, when changes in colony morphology were noted. Percentage of pathogen growth inhibition was calculated according to the formula by Siripornvisal (2010), i.e., $I\% = (r_o-r)/r_o \times 100$, where I% is percentage of growth inhibition; r_o is the radius of *F. graminearum* Fg1 colony on a control dish and r is the radius of *F. graminearum* Fg1 inhibited by the bacteria.

4.5.4. Identification of indigenous rhizosphere bacteria

The most promising isolates from section 4.5.3., i.e., those that changed pathogen growth morphology or those that inhibited *F. graminearum* Fg1 growth for more than 50%, were identified.

DNA of the isolates was extracted from overnight cultures grown in TSB (Tryptone Soya Broth; Carl Roth, Karlsruhe, Germany), using NucleoSpin R 96 Tissue kit (Macherey Nagel, Germany), according to the manufacturer's instructions. DNA concentration was determined using a UV Spectrophotometer (NanoPhotometer NP80, Implen, Munich, Germany).

Strains were further identified by amplifying the *rrs* gene encoding for 16S rRNA, using primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards et al., 1989). Each PCR reaction was done on a thermocycler Mastercycler (Eppendorf, Germany), in a volume of 50 µL, which contained: 5 µL of the buffer (10x DreamTaq Green Buffer with 20 mm $MgCl_2$; Thermo Fisher Scientific, USA), 5 µL of dNTP (2mM) (Thermo Fisher Scientific, USA), 0.25 µL of DreamTaq DNA polymerase (5 U/µL) (Thermo Fisher Scientific, USA), 2.5 µL of each primer (10 µM) (Thermo Fisher Scientific, USA), 50 ng of DNA and Rnase-free water up to 50 µL. Reaction conditions for primer pair pA/pH were as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 35 s, with a final elongation step at 72°C for 3 min. A mix of all the PCR reagents with Rnase-free water instead of DNA was used as a negative control, while a mix of all the PCR reagents with 50 ng of *P. fluorescens* F113 DNA served as a positive control. All of the PCR products were checked on 2% agarose gel (prepared with 0.5 x TBE buffer) to determine amplification success and relative band intensity. The amount of 5 µL of DNA Loading Dye (6x TriTrack, Thermo Fisher Scientific, USA) was mixed with 1 µL of each PCR product and deposited in agarose gel wells, and electrophoresis was run for 30 min at 100 V (Mupid-One, Advance Co. Ltd., Tokyo, Japan). PCR products were visualized under the UV lamp and compared to a DNA ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific, USA) and to the positive control which has the length of ~1500 bp for the *rrs* gene. Amplified fragments were sequenced with Sanger sequencing at Microsynth (Vaulx-en-Velin, France), in forward direction. Obtained *rrs* sequences were compared to the sequences available at the GenBank database (National Centre for Biotechnology Information, Bethesda, Maryland, USA) via Basic Local Alignment Search Tool (BLAST).

4.6. Analysis of rhizosphere *Pseudomonas* diversity through metabarcoding approach

As the genus *Pseudomonas* is known for its numerous mechanisms of pathogen suppression, as well as for its beneficial effects on the plant growth, the goal of this part of the experiment was to assess the diversity of fluorescent *Pseudomonas* in the rhizosphere of wheat plants grown in suppressive and non-suppressive MI soils. This analysis was done in two ways: through culture-independent method and through culture-based method. The first method was based on a metabarcoding approach, targeting the *rpoD* gene of the *P. fluorescens* group and the other method, explained in the section 4.7., was based on the isolation of putative *Pseudomonas* using the standard plating technique.

4.6.1. Isolation of rhizosphere DNA

The non-inoculated plants harvested at 28 days from section 4.3.2. were also used to assess root-associated *Pseudomonas* populations, using six rhizosphere replicates for each soil. Each root system was shaken to dislodge loosely-adhering soil and was flash-frozen in liquid nitrogen, followed by lyophilisation (for 24h, -50°C, 1.25 mbar; Lyophilizator, Alpha 1-4LSC, Christ, Germany). The root-adhering soil (i.e., rhizosphere soil) was separated from the roots using brushes and stored at −80°C, prior to DNA extraction using the FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch-Graffenstaden, France). DNA was extracted and eluted in 50 µL sterile ultra-pure water, according to the manufacturer's instructions, and DNA concentrations were determined using a UV Spectrophotometer (NanoPhotometer NP80, Implen, Munich, Germany).

4.6.2. *rpoD* sequencing from rhizospheric DNA

The *rpoD* gene coding for RNA polymerase sigma 70 (sigma D) factor was chosen to visualize diversity within the *Pseudomonas* genus. Primers with specific Illumina tails (rpoD_F: TCGCCAAGAAGTACACCAAC and rpoD_R: CCATGGAGATCGGCTCTT) (Manriquez, 2021) were used to amplify a 356 bp fragment of *rpoD*. PCR was done under the following conditions: 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 59°C for 40 s and 72°C for 45 s, with a final elongation step at 72°C for 10 min. PCR product purification, amplicon library construction, and Illumina MiSeq sequencing (2 × 300 bp paired-end reads) were performed by Microsynth.

4.6.3. Sequence data processing

Total reads obtained were demultiplexed. Reads quality was assessed using the software fastp v.0.23.2 (Chen et al., 2018) and primers were removed using the software cutadapt v.4.1 (Martin, 2011) with the default parameters. Then, the sequencing paired-end reads were processed using R software v.4.2.2 and the DADA2 package v.1.26 (Callahan et al., 2016) through a workflow step including filtering, trimming, denoising, dereplicating, merging and finally chimera removing. In the end, 928,217 reads were kept and distributed in 823 amplicon sequence variants (ASVs). Next, taxonomy was assigned using the DADA2 native implementation of the naïve Bayesian classifier method (Wang et al., 2007) and a home-made *rpoD* sequence database specific to the primer pairs. In conclusion, a total of 43 genera were identified in the microbial community, with 41 ASVs belonging to the *Pseudomonas* genus. The *rpoD* metabarcoding data were deposited into the EBI/EMBL database.

4.7. Formation of *Pseudomonas* collection

4.7.1. Isolation of *Pseudomonas*

Isolation of *Pseudomonas* was done in 96-well microplates, using the rhizosphere extracts (prepared as described in section 4.5.1.) of inoculated and non-inoculated soils i.e., MI2, MI3, MI4 and MI5 [i.e., 4 soils × 2 (inoculated/not with *F. graminearum* Fg1)], from the experiment described in section 4.3.2., according to a protocol by Vacheron et al. (2016). In brief, 20 µL of each prepared soil extract was mixed with 180 µL of King's B^{+++} [i.e., King's B supplemented with ampicillin (40 $\mu g.mL^{-1}$), chloramphenicol (13 $\mu g.mL^{-1}$) and cycloheximide (100 $\mu g.mL^{-1}$)] in microplates and then serially diluted, following a most probable number (MPN) design with five wells per dilution. Microplates were incubated at 28°C for 24h, and then 1 µL from the most diluted positive well was plated on King's B agar. Isolates were randomly picked for each of the eight conditions, purified and stored at -80°C in 25% glycerol.

4.7.2. Identification of *Pseudomonas*

Genomic DNA of all *Pseudomonas* isolates was extracted from overnight cultures grown in TSB, using NucleoSpin R 96 Tissue kit (Macherey Nagel, Germany), according to the manufacturer's instructions. DNA concentration was determined using a UV Spectrophotometer (NanoPhotometer NP80, Implen, Munich, Germany).

Identification was performed by sequencing the housekeeping gene *rpoD*, using primers rpoDf (5'-ACTTCCCTGGCACGGTTGACCA-3') and rpoDr (5'-TCGACATGCGACGGTTGATGTC-3')

targeting the *rpoD* alleles of bacteria from the *P. fluorescens* group (Frapolli et al., 2007). When *rpoD* amplification didn't succeed, the *rrs* gene encoding for 16S rRNA was amplified with pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') pair of primers (Edwards et al., 1989) and sequenced. Each PCR reaction was performed on a thermocycler Mastercycler (Eppendorf, Germany), in a volume of 50 µL, which contained: 5 µL of the buffer (10x DreamTaq Green Buffer with 20 mm $MgCl_2$; Thermo Fisher Scientific, USA), 5 µL of dNTP (2mM) (Thermo Fisher Scientific, USA), 0.25 µL of DreamTaq DNA polymerase (5 U/µL) (Thermo Fisher Scientific, USA), 2.5 µL of each primer (10 µM) (Thermo Fisher Scientific, USA), 50 ng of DNA and Rnase-free water up to 50 µL. Reaction conditions for primer pair rpoDr/rpoDf were as follows: 94°C for 150 s, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final elongation step at 72°C for 10 min, and for primer pair pA/pH as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 35 s, with a final elongation step at 72°C for 3 min. As a negative control, a mix of all the PCR reagents with Rnase-free water instead of DNA was used, while a mix of all the PCR reagents with 50 ng of *P. fluorescens* F113 DNA served as a positive control. All of the PCR products were checked on 2% agarose gel (prepared with 0.5 x TBE buffer) to determine amplification success and relative band intensity. The amount of 5 µL of DNA Loading Dye (6x TriTrack, Thermo Fisher Scientific, USA) was mixed with 1 µL of each PCR product and deposited in agarose gel wells, and electrophoresis was run for 30 min at 100 V (Mupid-One, Advance Co. Ltd., Tokyo, Japan). PCR products were visualized under the UV lamp and compared to a DNA ladder (GeneRuler DNA Ladder Mix, Thermo Fisher Scientific, USA) and to the positive control which has the length of ~700 bp and ~1500 bp for genes *rpoD* and *rrs*, respectively. Amplified fragments were sequenced with Sanger sequencing at Microsynth, in forward direction.

Obtained sequences were compared to the sequences available at the GenBank database (National Centre for Biotechnology Information, Bethesda, Maryland, USA), using BLAST. Analysis of sequences was done using the SeaView multiplatform (Gouy et al., 2010). The obtained sequences were aligned with MUSCLE v.3.8.31 (Edgar, 2022), they were manually filtered to discard gaps and aligned regions of low quality. Gblock software (Castresana, 2000; Talavera and Castresana, 2007) was used to eliminate poorly aligned positions, as well as divergent regions to prepare for phylogenetic analysis, and all duplicated sequences were discarded with seqkit software (Shen et al., 2016). The phylogenetic tree was constructed with Distance method and 1000 bootstraps and visualized using iTol (Letunic and Bork, 2021). Obtained gene sequences were deposited into the EBI/EMBL database.

4.8. Genome sequencing and genome annotation of selected biocontrol and *Pseudomonas* isolates

As genomic analysis of microbial isolates from fungistatic and non-fungistatic, suppressive and non-suppressive soils is useful to explore the underlying mechanisms of these phenomena, and considering that *Pseudomonas* isolates with biocontrol potential can also occur in non-suppressive soils (Ramette et al., 2006; Frapolli et al., 2010), the aim of this part of the study was to sequence the genomes of selected biocontrol and *Pseudomonas* isolates and to search for genes potentially involved in fungistasis and suppressiveness.

4.8.1. DNA extraction, genome sequencing and assembling

Genomic DNA extraction from chosen isolates was done from an overnight culture grown in TSB for 24h at 28°C, with 200 rpm (Innova 42R, New Brunswick Scientific, Edison, New Jersey, USA), using a Nucleospin tissue kit (Macherey-Nagel, France), according to the manufacturer's instructions. DNA quality and concentration were determined by agarose gel electrophoresis (1% gel concentration, 100 V, 30 min) (Mupid-One, Advance Co. Ltd., Tokyo, Japan), and with Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Genomic DNA was sequenced and library preparation was done at Novogene (England), using Illumina NovaSeq 6000 technology. The genomic DNA was randomly sheared into short fragments. The obtained fragments were end repaired, A-tailed and further ligated with Illumina adapter, generating a 2x150-bp paired-end library. The fragments with adapters were PCR amplified, size selected, purified and sequenced. The original data from Illumina platform were recorded in a FASTQ file, which contains sequencing reads and sequencing quality information. fastp software v.0.23.1 (Chen et al., 2018b) with default settings was used for trimming sequences and Unicycler software v.0.5.0 (Wick et al., 2017) with default settings for *de novo* assembly. Identification and construction of phylogenetic tree was performed with the Type Strain Genome Server (TYGS) (https://tygs.dsmz.de/; Meier-Kolthoff and Göker, 2019; Meier-Kolthoff et al., 2022). Genomic features of the isolates were obtained using the MicroScope platform (v.3.15.4; Vallenet et al., 2020). Whole-genome sequences (raw and assembled) were deposited into the EBI/EMBL database.

4.8.2. Genome annotation

Genome annotation was done automatically with the MicroScope platform. DIAMOND blastp (v.2.0.8.146; Buchfink et al., 2015) was used to search for genes involved in biocontrol and plant-growth promotion (query sequences are available in Supplementary material; Chapter 3; Table S1) within genome protein sequences, using the options --query-cover 80 --id 70, in order to filter the hits with minimum 80% query coverage and minimum 70% amino acid identity.

The searched functions included (i) production of antimicrobial compounds phenazine (*phzABCDEFG*) (Dar et al., 2020), HPR (*darABC*) (Nowak-Thompson et al., 2003), 2,4-diacetylphloroglucinol (*phlABCD*) (Bangera and Thomashow, 1999), pyrrolnitrin (*prnABCD*) (Kirner et al., 1998), HCN (*hcnABC*) (Ramette et al., 2003) and pyoluteorin (*pltABCDEFGLM*) (Nowak-Thompson et al., 1999), (ii) production of insect toxin FitD (*fitD*) (Loper et al., 2012) and alkaline metalloproteinase AprA (*aprA*) (Loper et al., 2012) involved in biocontrol, (iii) production of siderophores pyoverdine (*pvdL*) (Schalk and Guillon, 2013), pyochelin (*pchABCDEF*) (Reimmann et al., 2001) and pseudomonine (*pmsABCE*) (Matthijs et al., 2009), (iv) signaling and modulation of plant hormonal balance by deamination of ethylene precursor ACC (*acdS*) (Shah et al., 1998), ethylene production (*efe*) (Wang et al., 2010), auxin biosynthesis (*iaaMH*, *ipdC*/*ppdC*) (Loper et al., 2012; Gruet et al., 2022), auxin catabolism (*iacABCDEFGHI*) (Loper et al., 2012), acetoin synthesis (*budB*/*ilvNB*/*alsS*, *budA*/*alsD*) (Blomqvist et al., 1993; Loper et al., 2012), 2,3-butanediol synthesis (*budC*/*ydjL* in addition to the acetoin synthesis genes) (Nicholson, 2008), 2,3-butanediol conversion to acetoin (*adh*/*bdhA*/*ydjL*) (Huang et al., 1994; Nicholson, 2008), acetoin catabolism (*acoABCX*) (Huang et al., 1994), (v) transformation of P and N sources by phosphate solubilization (*gcd*, *gad*) (Miller et al., 2010), nitrogen fixation (*nifHDK*) (Bruto et al., 2014) and denitrification (*nirK*, *nirS*) (Coyne et al., 1989; Bruto et al., 2014). In case where presence of more than one gene is necessary to achieve a function (e.g., presence of both *iaaM* and *iaaH* genes for the synthesis of auxin via the indole-3-acetamide

pathway), but only some of the necessary genes were found in the genome, the presence of the missing genes was checked with less stringent BLAST result filtering criteria (--query-cover 80 --id 30). Putative biosynthetic gene clusters were further identified using the antiSMASH (Blin et al., 2019) within the MicroScope platform and the annotations were manually curated.

Carbohydrate-active enzymes (CAZymes) were predicted using dbCAN2 v.3 (Zhang et al., 2018) and compared with the CAZy database using HMMER v.3.3 (Eddy, 2011). Prediction of function and substrate specificity of CAZyme families or subfamilies was performed based on a review of activities assigned to CAZymes with known structures (characterized enzymes) in the CAZy database (http://www.cazy.org) (Lombard et al., 2014) and manually curated, as previously described (López-Mondéjar et al., 2022). A heatmap based on CAZyme counts was generated by pheatmap v.1.0.12 package (Kolde, 2019).

4.9. Plant growth promoting (PGP) characterization of biocontrol and *Pseudomonas* isolates

Functional characterization of isolates included the assessment of both direct biocontrol and indirect PGP mechanisms. Biocontrol mechanisms included: siderophore production, production of HCN and production of lytic enzymes (proteases, chitinases and cellulases), while PGP mechanisms included: phytohormones production, ACC deaminase production and solubilization of phosphates. Additionally, the inhibitory effect of VOCs produced by rhizosphere isolates towards *F. graminearum* Fg1, as well as the ability of bacterial isolates to inhibit conidia germination of *F. graminearum* Fg1 in liquid medium, were tested.

4.9.1. Siderophores production

Siderophores production was tested on Chrome Azurol S (CAS) agar according to an adapted protocol by Lakshmanan et al. (2015). In brief, 60.5 mg of CAS was dissolved in 50 ml of water and mixed with 10 ml of $FeCl_3$ solution (1 mM $FeCl_3$ x $6H_20$ in 10 mM HCL). Then, 72.9 mg of hexadecyltrimethylammonium (HDTMA; Sigma-Aldrich, St Louis, MO, USA) was added to 40 ml of water, slowly mixed with CAS-$FeCl_3$ solution and then autoclaved for 20 min at 121°C. Separately, 15% agar solution was prepared and autoclaved for 20 min at 121°C, cooled down to 50°C and mixed with CAS-$FeCl_3$-HDTMA media. As HDTMA often inhibits growth of Gram-positive bacteria, O-CAS (overlaid CAS) method developed by Pérez-Miranda et al. (2007) was further used. Isolates were firstly grown in triplicates on LB agar (pH 6.5) (Luria-Bertani; tryptone, 10 g; NaCl, 10 g; yeast extract, 5 g; agar, 15 g; H_20, 1000 ml) by inoculating 2 µL of each bacterial suspension and incubating for 24 h at 28°C. After the incubation period and development of colonies, 10 ml of the prepared CAS agar was overlayed on top of LB plates with grown colonies. After 24h, the change of media colour around the colonies from blue to orange was assessed as an indication of the siderophores production.

4.9.2. Production of hydrogen cyanide

Isolates' ability to produce HCN was tested according to a protocol by Bakker and Schippers (1987). Bacterial isolates were plated in triplicates on TSA plates supplemented with 4.4 g glycine.L^{-1} and the plates were inverted. Sterile filter paper was impregnated with 0.5% picric acid and 2% Na_2CO_3 and it was placed in the lid of each plate. Plates were firmly sealed with

parafilm and incubated at 28°C for 96 h. Change of the colour of the filter paper from yellow to orange-brown after incubation indicated production of HCN by the isolates.

4.9.3. Production of lytic enzymes (proteases, chitinases and cellulases)

Protease activity was tested on sterile Milk agar, prepared by mixing one volume of pasteurized skim milk with one volume of 2 % agar solution previously autoclaved for 20 min at 121°C. Medium was inoculated with 2 µL of each bacterial suspension, previously grown in TSB overnight, in triplicates, and incubated at 28°C for 48h. After the incubation period, presence of halozones around the colonies indicated the production of proteases by the isolates.

Chitinase activity was tested on Minimal media (KH_2PO_4, 0.7 g; K_2HPO_4, 0.3 g; NaCl, 4 g; $MgSO_4$ x $7H_2O$, 0.5 g; $FeSO_4$ x $7H_2O$, 1 mg; $ZnSO_4$ x $7H_2O$, 0.1 mg; $MnSO_4$ x $7H_2O$, 0.1 mg) (Kim et al., 2003) supplemented with 10 % colloidal chitin solution, prepared as described by Murthy and Bleakley (2012). Prepared media were inoculated with 2 µL of each bacterial suspension, previously grown in TSB overnight, in triplicates, and incubated at 28°C for seven days. After seven days, the appearance of colonies indicated their possibility of using chitin as a carbon source, showing the chitinase activity.

Production of extracellular cellulases was tested on media containing Carboxymethyl cellulose (CMC; Sigma Aldrich, USA), of the following composition: CMC, 10 g; K_2HPO_4, 5 g; $MgSO_4$ x $7H_20$, 0.25 g; $CaCl_2$, 0.2 g; $MnSO_4$, 0.1 g; $NaNO_3$, 2 g; $FeSO_4$, 0.01 g; agar, 20 g; H_20, 1000 ml. This assay was performed in triplicates, by inoculating 2 µL of each bacterial suspension, previously grown in TSB overnight, on CMC media and incubating for 48 h at 28°C. After the incubation period, plates were flooded with 0.1% Congo red solution in 1M NaCl. After 10 min, the dye was rinsed with 1M NaCl. The appearance of halozones around the colonies indicated cellulose activity.

4.9.4. Production of phytohormones

Screening for the production of (i) seven auxin phytohormones, i.e., IAA, indole-3-lactic acid, indole-3-carboxylic acid, indole-3-pyruvic acid, indol-3-butyric acid (IBA), tryptophol and indole-3-propionic acid, (ii) five cytokinins, i.e., trans-zeatin, trans-zeatin riboside (ZR), kinetin, 6-benzylaminopurine (BAP) and isopentenyl adenosine (IPA), (iii) two gibberellins, i.e., gibberellin A1 (GA1) and gibberellic acid (GA3), (iv) abscisic acid (ABA) and (v) kynurenic acid was done by Ultra High Performance Liquid Chromatography (UHPLC) at the CESN platform (UMR5557, University Lyon1). Briefly, all isolates were grown three days at 28°C (300 rpm) in 2 mL of M9 minimal medium (Miller, 1972) supplemented with 0.4 mM of tryptophan and 0.1 mM of adenine. The cultures were centrifuged at 4500*xg* during 8 min and filtered at 0.2 µm. Supernatants were lyophilized (for 24h, -50°C, 1.25 mbar; freeze-dryer, Lyophilizator, Alpha 1-4LSC, Christ, Germany), the powder obtained was extracted two times with methanol, drying with speed-vac (Centrivap Cold Trap Concentrator LABCONCO, Kansas City, MO, USA), and UHPLC separation was performed with an Agilent 1290 Series instrument (Agilent Technologies France, Les Ulis, France) using a 100 × 3 mm reverse phase column (Agilent Poroshell 120 EC-C18, 2.7 µm particle size). Samples (3 µL) were loaded onto the column equilibrated with solvent A (water + 0.4% formic acid) and solvent B (acetonitrile) in a 98:2 ratio. Compounds were eluted by increasing the acetonitrile concentration to 40% over a 6 min period, then to 100% over 4 min, followed by an isocratic step of 2 min, at a flow rate of 0.5 $mL.min^{-1}$. Hormones were detected with a diode array detector (DAD) and an Agilent 6530 Q-TOF mass spectrometer in

positive and negative electrospray ionization, based on comparison with commercial standards on both mass and UV (between 190 and 600 nm) chromatograms, along with accurate mass and UV spectra.

4.9.5. 1-aminocyclopropane-1-carboxylate deaminase production

Production of ACC deaminase was tested according to a modified protocol by Penrose and Glick (2003), which detects α-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. Bacterial cultures were firstly grown in 15 ml of TSB for 24h at 28°C, shaking at 200 rpm (Innova 42R, New Brunswick Scientific, Edison, New Jersey, USA). After the incubation, the cultures were centrifuged at 8000x*g* for 10 min (Avanti J-E Series, Beckman Coulter, Fullerton, USA), supernatant was removed and bacterial cells were washed twice with DF salts minimal medium (Dworkin and Foster, 1958; KH_2PO_4, 4 g; Na_2HPO_4, 6 g; $MgSO_4$ x $7H_2O$, 0.2 g; glucose, 2 g; gluconic acid, 2 g and citric acid, 2 g with trace elements: $FeSO_4$ x $7H_2O$, 1 mg; H_3BO_3, 10 mg; $MnSO_4$ x H_2O, 11.19 mg; $ZnSO_4$ x $7H_2O$, 124.6 mg; $CuSO_4$ x $5H_2O$, 78.22 mg; MoO_3, 10 mg; H_20, 1000 ml; pH 7.2), and finally resuspended in 7.5 ml DF salts minimal medium, with addition of 45 µL of 0.5 M ACC solution (0.5 M ACC solution was filter-sterilized at 0.2 µm, aliquoted, frozen at -20 °C and thawed prior to the addition). Bacterial cultures were incubated once again for 24h at 28°C, with shaking at 200 rpm, cells were harvested by centrifugation at 8000x*g* for 10 min and washed twice with 0.1 M Tris-HCl, pH=7.6. The pellet was resuspended in 600 µL of 0.1 M Tris-HCl, pH=8.5, lysed with 30 µL of toluene and shaking for 1 min in a MM200 Retsch mixer mill (Bioblock, Vaulx Milieu, France) to ensure complete cell lysis. Then, 200 µL of the toluenized cells were transferred to fresh 1.5-ml microcentrifuge tubes and 20 µL of 0.5 M ACC was added to the suspension, vortexed, and then incubated at 30°C for 15 min. Following the addition of 1 ml of 0.56M HCl, the mixture was vortexed and centrifuged for 10 min at 8000x*g* at room temperature. One millilitre of the supernatant was vortexed together with 800 µL of 0.56 M HCl and 300 µL of the 2,4-dinitrophenylhydrazine reagent (0.2% 2,4- dinitrophenylhydrazine in 2 M HCl) and then incubated at 30°C for 30 min, during which time the α-ketobutyrate was transformed to phenylhydrazone. The colour of the phenylhydrazone was developed by the addition of 2 ml of 2 M NaOH and compared to the colour of assay reagents in the presence of ACC, without the bacterial extracts. Colour of the control was pale yellow, and bacterial isolates positive for the production of ACC deaminase developed brown-red colour.

4.9.6. Phosphate solubilization

Isolates' ability to solubilize inorganic phosphates was tested on NBRIP media (National Botanical Research Institute's Phosphate; Nautiyal, 1999) of the following composition: glucose, 10 g; $MgCl_2$ x $6H_20$, 5 g; $MgSO_4$ x $7H_20$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g; $Ca_3(PO_4)_2$, 5 g; agar, 20 g; H_20, 1000 ml; pH=7. Each assay was performed in triplicates by inoculating 2 µL of each bacterial suspension, previously grown in TSB overnight, on NBRIP media, and incubating for 14 days at 28°C in the dark. After the incubation period, presence of halozones around the colonies indicated the solubilization of phosphates by the isolates.

4.9.7. The effect of bacterial volatile organic compounds (VOCs) on *Fusarium graminearum* Fg1 growth

The inhibitory effect of VOCs produced by bacterial isolates towards *F. graminearum* Fg1 was assessed in a system of two Petri dishes sealed together with parafilm. For this assay, each bacterial isolate was grown in TSB at 28°C/24h/200 rpm (Innova 42R, New Brunswick Scientific, Edison, New Jersey, USA). Afterwards, the optical density of obtained suspension was adjusted to 1 (i.e., 10^8 cells.mL^{-1}) at 600 nm (OD600nm, Ultrospec 10 Cell Density Meter; Amersham Biosciences, Little Chalfont, UK) and then 30 µL of this prepared suspension was spread onto a TSA plate. PDA plates were center-inoculated with discs (Ø7 mm) taken from the edges of 8-days old *F. graminearum* Fg1 colony. After 24 h of bacterial and fungal growth, at 28°C and 22°C, respectively, the lid of TSA plate with bacteria was replaced with a PDA plate containing *F. graminearum* Fg1 and the two plates were firmly sealed together with parafilm. Each assay was done in triplicate. Control plates were prepared in the same way, but without the bacteria in the bottom plate. Such sealed plates were incubated at 22°C, and the observations were recorded after 72 h. The mycelial growth inhibition (%) of the fungus was determined according to Trivedi et al. (2008), using the formula $(1-r_2/r_1) \times 100$, where r_1 represents the radial growth of *F. graminearum* Fg1 in control plates, and r_2 in plates with bacteria.

4.9.8. *Fusarium graminearum* Fg1 spore germination inhibition test in liquid medium

Antagonism potential of bacterial isolates on *F. graminearum* Fg1 spore germination, was tested in a microplate test, according to a protocol by Besset-Manzoni et al. (2019). Each tested bacteria was grown in TSB for 24h, with shaking at 200 rpm (Innova 42R, New Brunswick Scientific, Edison, New Jersey, USA), centrifuged at 6000x*g* for 10 min (Avanti J-E Series, Beckman Coulter, Fullerton, USA) and supernatants were filtered at 0.2-µm. *F. graminearum* Fg1 macroconidia were prepared by growing the fungus in MBB. For each assay, 100 µL of the prepared bacterial supernatant, 100 µL of PDB (Potato Dextrose Broth, Condalab), and 50 µL of *F. graminearum* Fg1 spores at 10^4 spores.mL^{-1} were added in microplate wells, in triplicates. For positive control, 100 µL of TSB was used instead of bacterial supernatants, and for negative control, 50 µL of PDB was used instead of *F. graminearum* Fg1 spore suspension. After incubating microplates for 5 days at 28°C, the turbidimetry was measured at 492 nm using an Infinite M200 Pro microplate reader (TECAN, Mannedorf, Switzerland), the value of negative control was subtracted from each bacterial treatment and compared to the positive control.

4.10. *In planta* protection assay with chosen biocontrol and *Pseudomonas* isolates

In this part of the study, chosen indigenous rhizosphere bacteria with biocontrol properties and *Pseudomonas* were used to perform *in planta* biocontrol assay, in the presence of *F. graminearum* Fg1, aiming to assess the phytoprotective capacity of these isolates.

4.10.1. Preparation of *Fusarium graminearum* Fg1 spore suspension and bacterial suspensions

F. graminearum Fg1 spore suspension was prepared by growing the fungus in MBB, as described in section 4.3.1., and adjusted to 10^6 spores.mL^{-1}. For each *in vivo* test, 24h-old bacteria were prepared by growing in TSB at 28 °C/200 rpm (Innova 42R, New Brunswick Scientific, Edison,

New Jersey, USA). Cultures were centrifuged at 6000x*g* for 10 min (Avanti J-E Series, Beckman Coulter, Fullerton, USA), supernatant was discarded and bacterial cells were washed twice with 10 mM $MgSO_4$. Finally, bacterial suspension was prepared by resuspending bacterial pellets in 10 mM $MgSO_4$ and adjusting optical density at 600 nm to 1 (Ultrospec 10 Cell Density Meter, Amersham Biosciences, Little Chalfont, UK).

4.10.2. *In planta* protection assay

The *in planta* protection assay was carried out in a plant growth chamber (FitoClima, 10.000 EH, ARALAB, Rio de Mouro, Portugal), under the following conditions: 16h day at 20°C/8 h dark at 18°C and 80 % relative humidity. Chosen biocontrol and *Pseudomonas* isolates were tested in soil without documented suppressiveness, taken in La Côte-Saint-André, France (sampled as described in section 4.1.; Bouffaud et al., 2016). For each treatment, 30 seeds of wheat cultivar Sumai 3 were distributed in 10 pots (8 x 6 x 6 cm), each filled with 150 g of sterile LCSA soil (autoclaved twice at 121°C for 20 min, 24h interval). Bacteria were inoculated directly onto each seed (10^7 cells per seed) with 100 µL of prepared suspensions. The same day, 100 µL of *F. graminearum* Fg1 spores (10^5 spores per seed) were added directly onto seeds. As a negative control, 30 uninoculated seeds were used, while 30 seeds inoculated only with *F. graminearum* Fg1 spores served as a positive control. Plants were watered every three days by adding the water under the pots (watering by capillary movement of water).

At 14 days, the number of germinated seeds was counted. After 45 days of the plant growth, the plants were harvested and measurements were performed, such as: (i) shoot biomass (mg), (ii) the chlorophyll rate of each wheat plant containing three measurements of the 5^{th}, 6^{th} and 7^{th} grown leaf using the SPAD 502 plus device (Minolta Camera Co., Osaka, Japan) and (iii) the disease symptoms of crown-rot on each wheat collar using a 1 to 7 notation index, as follows: 1 = no symptoms, 2 = several non-connected, dark spots only at the collar base, 3 = several non-connected, dark spots, rising up the collar base, 4 = several connected, dark spots, 5 = several connected, dark spots, rising up to several cm, 6 = collar covered with necrosis, collar base very fragile, and 7 = dead plant.

4.11. Methods used for identifying novel species

TYGS whole-genome phylogenetic analysis of *Pseudomonas* isolates (from the section 4.7.), whose genomes were fully sequenced (as described in section 4.8.1.), pointed to the existence of novel species. As required when describing novel species, ANI analysis was performed, to confirm that the obtained ANI values between novel species and the closest described species present in the public databases are below 95% (Chun et al., 2018). The selected strains were described phenotypically and phylogenetically, and were deposited in three culture collections, i.e., Collection Française de Bactéries associées aux Plantes (CFBP), Belgian Coordinated Collections of Microorganisms (BCCM/ LMG) and Environmental Microbiology Lyon - Biological Resource Centre (EML-BRC, https://brclims.pasteur.fr/brcWeb/souche/ recherche), as required by International Journal of Systematic and Evolutionary Microbiology (microbiologyresearch.org). Their whole-genome sequences (raw and assembled) were deposited into the EBI/EMBL database.

4.11.1. Phylogenetic analyses

TYGS was used to construct phylogenetic trees based on whole-genome and *rrs* sequences of the chosen strains and other *Pseudomonas* type strains present in the database. Briefly, the TYGS pipeline selects the closest type-strain genomes using two complementary ways. First, the genomes that were assessed ('query') were compared with all available type-strain genomes in the TYGS database using the MASH algorithm, a fast approximation of intergenomic relatedness (Ondov et al., 2016), and the type strains with the lowest MASH distances per requested genome were selected. Second, the *rrs* sequences were used to identify an additional set of closely-related type strains. *rrs* sequences were extracted from the studied genomes using RNAmmer (Lagesen et al., 2007) and each *rrs* sequence was then blasted (Camacho et al., 2009) against the 18,799 type strains available in the TYGS database. This dual approach was used to find the 50 best matching type strains (based on the binary score) for each user genome, and then to calculate accurate distances using the Genome BLAST Distance Phylogeny (GBDP) method based on the coverage algorithm and the d5 distance formula (Meier-Kolthoff et al., 2022). These distances were then used to determine the 10 closest type-strain genomes for each query genome. The *rrs* phylogenetic tree was inferred with FastME v.2.1.6.1 (Lefort et al., 2015) based on GBDP distances (Meier-Kolthoff et al., 2022). However, as only poor resolution of strains is often achieved with *rrs* sequences (Rodriguez-R et al., 2018), a phylogenetic tree with whole-genome sequences was also inferred, using FastME 2.1.6.1 (Lefort et al., 2015) and GBDP distances calculated from whole genome sequences. The trees were visualized using iTOL software (Letunic and Bork, 2021). The genomic relatedness of the studied strains to the type strains available in public databases was ascertained by calculating the ANI and digital DNA–DNA Hybridization (dDDH) values. ANI by BLAST (ANIb) was calculated using the JSpecies server, based on BLAST (Richter et al., 2016) and dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0) (Meier-Kolthoff and Göker, 2019; Meier-Kolthoff et al., 2022), using the recommended BLAST method. The GGDC results were based on formula 2, which is independent of the genome length and is recommended for incomplete draft genomes. Recommended cut-off values for ANI and dDDH for prokaryotic species differentiation are 95–96% and 70%, respectively (Chun et al., 2018). Percentage of 16S rRNA gene identity of studied strains with the closest type strains available in the public databases was calculated using the EzBioCloud server (Yoon et al., 2017). Additionally, pan-genome analysis was performed with the MicroScope platform (amino acid identity > 80%; alignment coverage > 80%) (Vallenet et al., 2020) to visualize the core and unique genes between strains of the same species presented in this study.

4.11.2. Morphological, biochemical and physiological characterization

Gram staining of the chosen isolates was done with standard methods. Temperature range was determined by growing strains on TSA at 4, 10, 37 and 41°C for 48 h. The range of pH suitable for growth was determined by inoculating Nutrient Broth (Condalab, Madrid, Spain) with pH adjusted to 3, 4, 5, 6, 7, 8 and 9 and incubating for 48 h at 28°C. Oxidase activity was assessed using Oxidase test strips (Sigma Aldrich, St Louis, MO, USA) and catalase activity by resuspending one colony in a drop of 3% hydrogen peroxide and monitoring bubble production. Fluorescent pigment production was tested on King's B agar, Pseudomonas Agar F (PAF; BD Difco, Sparks, MD, USA) and PDA. Swimming, swarming and twitching motilities were checked by stab-inoculating media containing 0.3% meat extract, 0.5% peptone, 0.5% NaCl as well as 0.3%,

0.5% and 1.5% agar (pH 7), respectively, in triplicates. In brief, bacterial strains were grown overnight in TSB, 2 mL of cell suspension was transferred to 2 mL tube, centrifuged at 8000x*g* for 10 min, after which the supernatant was discarded. The pellet was stabbed with the tip of a sterile toothpick, inoculated in the middle of the plates containing different concentrations of agar and incubated for 48 h at 28°C. Physiological characterization was performed with Biolog GEN III MicroPlates (Biolog, Hayward, CA, USA), API 20 NE and API ZYM strips (BioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions. Further testing included phenotypic characterization described in section 4.9.

4.11.3. Antibiotic susceptibility

Strains in this study were tested for their susceptibility to 10 different antibiotics using the disc diffusion method (Bauer et al., 1966). The antibiotic discs (Bio-Rad, Marnes-la-Coquette, France) included imipenem (10 µg), cefepime (30 µg), amikacin (30 µg), ticarcillin (75 µg), ticarcillin/clavulanic acid (75 + 10 µg), tobramycin (10 µg), meropenem (10 µg), aztreonam (30 µg), ciprofloxacin (5 µg) and levofloxacin (5 µg). In brief, the antibiogram was done by plating bacterial suspension (density at 625 nm adjusted to 0.5 McFarland units with sterile saline solution) on Mueller Hinton medium (MH; Condalab) and firmly applying antibiotic disks on the agar surface. Inverted plates with antibiotic discs were incubated for 24 h at 33°C (as recommended by EUCAST and SFM, Manual v.1.0. May 2022, CASFM2022_V1.0.pdf; sfm-microbiologie.org). After incubation, antibiotic sensitivity or resistance was evaluated by measuring zones of inhibition and comparing to critical values for *Pseudomonas aeruginosa* available at European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org).

4.12. Statistical analyses

All the data were analyzed at $P < 0.05$, using the R 4.2.1. software (https://www.r-project.org).

4.12.1. Fungistasis assay

For qPCR data, outliers were detected using the Grubbs' test (Grubbs, 1969; Burns et al., 2005) and discarded. qPCR data are presented as means ± standard errors. Firstly, these data were processed using an ANOVA, followed by Fisher's LSD tests from the *agricolae* package (de Mendiburu, 2023), to assess differences in fungistasis levels for 26 fields. Secondly, a two-way ANOVA was performed to assess the effects of field location × manure amendments. Thirdly, differences between manured vs. non-manured fields at all five locations were tested with ANOVA and LSD tests. Finally, Chi^2 tests were used to assess the relationship between manure amendments and fungistasis.

4.12.2. Suppressiveness assay

The greenhouse suppressiveness experiment followed a randomized block design with 10 replicates (i.e., 10 pots). When the data did not display normal distribution and homogeneity of variance, based on Shapiro and Levene tests, respectively, Kruskal-Wallis and post-hoc Dunn's tests were used. When the data displayed normal distribution and homogeneity of variance, an ANOVA followed by Tukey's HSD tests was used. Additionally, for plant shoot length, shoot

biomass and density, t tests were performed to compare plants grown in manured vs. non-manured soils. For shoot length, biomass and density, the plants that did not germinate were regarded as missing data (NA). Results are presented as means ± standard errors. For each plant growth parameter, letters a-c show the statistical relationship between the soils and treatments.

4.12.3. 16S rRNA gene and ITS sequencing

For the microbial communities in suppressive vs. non-suppressive soils, samples with low number of reads or ASVs were discarded. Specialized R package functions were used to determine taxa relative abundances, alpha and beta diversities and to perform statistical tests. Alpha diversity was computed, and sequences were rarefied based on the lowest number of sequences identified among samples, with a minimum of 41,961 sequences for 16S rRNA gene and 34,482 sequences for ITS. Alpha diversity indices were computed for each rarefied sample using the phyloseq (McMurdie and Holmes, 2013), microbiome (Lahti et al., 2018), or vegan (Oksanen et al., 2022) packages. Kruskal–Wallis tests were used to assess changes in alpha diversity with 10,000 permutations. If the Kruskal–Wallis test led to rejecting the null hypothesis ($P < 0.05$), LSD tests with Bonferroni correction were conducted to compare categories using *agricolae* package (de Mendiburu, 2023). Kruskal–Wallis tests were also used to assess the effect of inoculation on the relative abundance of phyla.

Beta diversity analysis in suppressive vs. non-suppressive soils was carried out using the rarefied datasets and the ASVs for both 16S rRNA gene and ITS. The dissimilarity among samples was determined by calculating the Bray-Curtis distance. The statistical significance of the comparisons was assessed using a permutation analysis of variance (PERMANOVA) with 10,000 permutations using the adonis2 function of the vegan (Oksanen et al., 2022) package. Non-metric multidimensional scaling (NMDS) was employed to visually represent the microbial communities with the ggplot2 (Wickham, 2011) package. Analysis of similarities (ANOSIM) (with 10,000 permutations) was used to compare microbial communities of the three soils (MI2, MI4 and MI5), while pairwise comparisons were used for pairwise comparisons of microbial communities (for MI5 vs. MI4, MI5 vs. MI2, and MI4 vs. MI2).

A negative binomial Wald test implemented in DESeq2 v.1.18.1 within the phyloseq R package was employed to identify taxa with significant differences to test for differential abundance (DA) on unrarefied reads (Love et al., 2014) in suppressive vs. non-suppressive soils. After the Benjamini-Hochberg correction method, the taxa were considered differentially abundant when the adjusted *P* value was below 0.05. Control was tested against *Fusarium* inoculation (Fg1 samples) for each soil.

For the analysis of *Fusarium* diversity in suppressive vs. non-suppressive soils, all ASVs affiliated with the genus *Fusarium* were kept. When possible, the taxonomic identification at the species level was used, based on the UNITE database (Nilsson et al., 2019). In each soil and inoculation condition, the proportion of *Fusarium* reads among the total number of fungal reads was computed, as well as the proportion of reads for each identified *Fusarium* species among the total number of *Fusarium* reads. To assess the impact of Fg1 inoculation on the abundance of each identified *Fusarium* species, ASV data from the eleven retrieved *Fusarium* species were treated with Kruskal-Wallis tests, followed by post-hoc LSD tests with Benjamini-Hochberg correction ($P < 0.05$).

4.12.4. *rpoD* sequencing

For *rpoD* microbiota analysis, the packages phyloseq (McMurdie and Holmes, 2013), vegan (Dixon, 2003) and ade4 (Dray and Dufour, 2007) were used. Alpha diversity analysis was performed by computing the index of observed richness and Chao1 for richness (Chao, 1987), and Shannon (Shannon, 1948) and inverse Simpson (Simpson, 1949) for diversity and evenness. Relationships between soil and the presence/absence of *Pseudomonas* were evaluated using the envfit procedure of the package vegan. Graphs and figures were plotted using the package ggplot2 (Wickham, 2011).

4.12.5. Greenhouse phytoprotection assay with chosen biocontrol and *Pseudomonas* isolates

The greenhouse phytoprotection experiment followed a randomized block design, with 10 replicates (i.e., 10 pots). When the data did not display normal distribution and homogeneity of variance, based on Shapiro and Levene tests, respectively, Kruskal-Wallis tests and post-hoc Dunn's tests were used to compare treatments. When the data displayed normal distribution and homogeneity of variance, an ANOVA followed by Tukey's test was used. For biomass, symptoms and chlorophyll content, the plants that did not germinate were regarded as missing data (NA). All results were presented as mean + standard error. For each plant growth parameter, letters a-d are used to show the statistical relationship between the treatments.

5. RESULTS

5.1. Field survey

The first objective of this research was to identify soils fungistatic and suppressive to *F. graminearum*, to investigate the relation between manure amendments and the occurrence of fungistasis/suppressiveness and to compare chosen soils based on their prokaryotic and fungal rhizosphere diversity.

To achieve the first objective, 26 agricultural fields (with or without manure amendments) were sampled, from two contrasting regions in Serbia: (i) in the northern plains region of Serbia (near Sombor (SO) and Novi Karlovci (NK)), where the agriculture is more intensive, and (ii) in the western and central hilly region of Serbia (near Valjevo (VA), Mionica (MI) and Čačak (CA)), where the agriculture is less intensive (Figure 7AB).

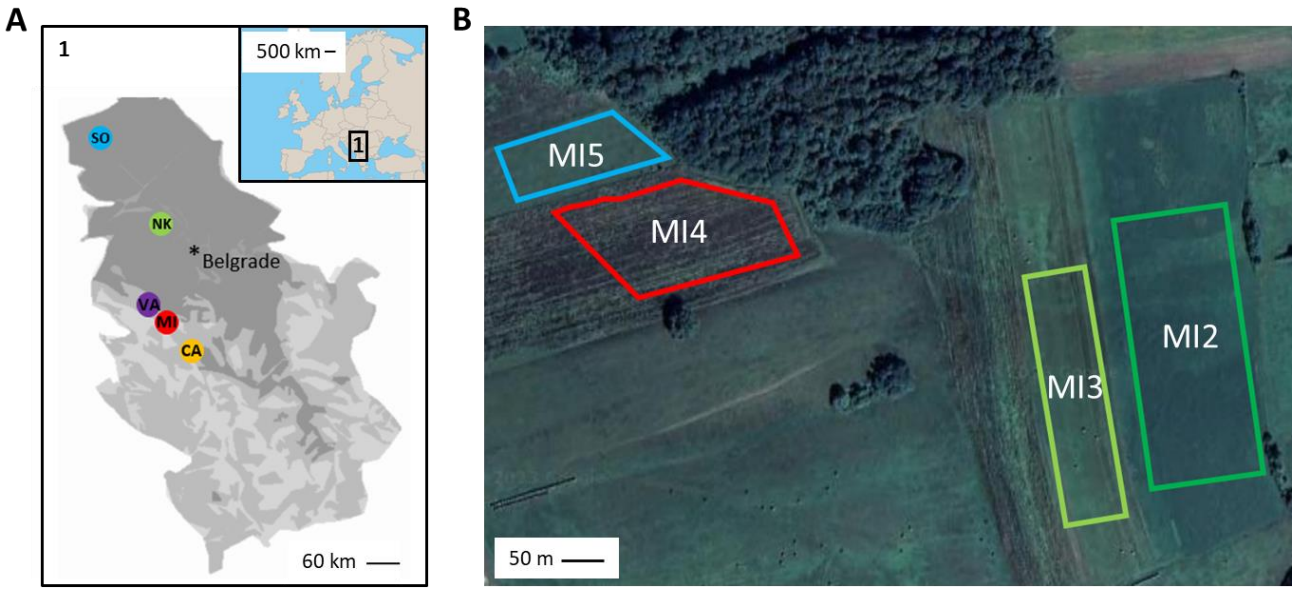

Figure 7. Soil sampling locations in Serbia. (A) Five locations in Serbia, i.e., Sombor (SO), Novi Karlovci (NK), Valjevo (VA), Mionica (MI) and Čačak (CA) where the soils from 26 fields were sampled. (B) Aerial picture of the four sampling fields MI2, MI3, MI4 and MI5 near Mionica, Serbia, visualized in Google Maps [Map data ©2023, Google].

At each of these fields, wheat was grown in rotation, and at each location, soils were sampled from fields in a close proximity, that previously had or had not received manure. At each of these locations, the farmers filled in the questionnaire about the manure amendments (type and quantity, if applicable), recent cropping history, fertilizers and pesticides application, management of postharvest residues and the observed presence of wheat fusariosis. Summary of farmers' questionnaire responses are presented in Table 5. Soil type at each of these fields was determined according to the pedological maps by Tanasijević et al. (1964) and Nejgebauer et al. (1971).

Table 5. Locations, sample ID, soil type, type and quantity (t/ha) of manure, recent cropping field history, use of fertilizers and pesticides, postharvest residues management and the observed presence of wheat fusariosis of 26 Serbian soils.

Location	Sample ID	Soil type*	Type of animal manure	Manure quantity	Recent cropping history (most recent is first)	Use of mineral fertilizers	Use of pesticides	Postharvest residues management	Observed presence of wheat fusariosis in recent years
Sombor	SO1	Chernozem	-	-	Wheat-soybean-maize	Yes	Yes	Ploughing	Yes
	SO2	Chernozem	-	-	Wheat-soybean-maize	Yes	Yes	Ploughing	Yes
	SO3	Chernozem	Beef	35 t/ha	Wheat-maize-wheat-maize	Yes	Yes	Ploughing	Yes
	SO4	Chernozem	Beef	35 t/ha	Wheat-maize-wheat-maize	Yes	Yes	Ploughing	Yes
Novi Karlovci	NK1	Chernozem	Beef	14.5 t/ha	Wheat-maize-sunflower-beetroot	Yes	Yes	Ploughing	No
	NK2	Chernozem	Beef	14.5 t/ha	Wheat-maize-sunflower-beetroot	Yes	Yes	Ploughing	No
	NK3	Chernozem	-	-	Wheat- sunflower-beetroot-maize-beetroot	Yes	Yes	Ploughing	No
	NK4	Chernozem	-	-	Wheat- sunflower-beetroot-maize-beetroot	Yes	Yes	Ploughing	No
Valjevo	VA1	Eutric cambisol	Sheep, beef and chicken	70-80 t/ha	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	No
	VA2	Eutric cambisol	-	-	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Burning	No
	VA3	Eutric cambisol	-	-	Oat-wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	No
	VA4	Eutric cambisol	Sheep, beef and chicken	70-80 t/ha	Maize-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	No
	VA5	Pseudogley	-	-	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing and burning	No
	VA6	Pseudogley	Beef	80 t/ha	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing and burning	No

	VA7	Pseudogley	Beef	80 t/ha	Maize-wheat-maize-wheat-maize	Yes	Yes**	Ploughing	No
	VA8	Pseudogley	-	-	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	No
Mionica	MI2	Vertisol	Sheep	80 t/ha	Alfalfa-wheat-maize-wheat-maize	Yes	Yes**	Ploughing	No
	MI3	Vertisol	Sheep	80 t/ha	Sunflower-wheat-maize-wheat-maize	Yes	Yes**	Ploughing	No
	MI4	Vertisol	-	-	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	No
	MI5	Vertisol	-	-	Meadow-wheat-maize-wheat-maize	Yes	Yes**	Ploughing	No
Čačak	CA1	Vertisol	-	-	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	Yes
	CA2	Vertisol	Beef	30-40 t/ha	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	No
	CA3	Vertisol	-	-	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	Yes
	CA4	Vertisol	Beef	30-40 t/ha	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	No
	CA5	Vertisol	-	-	Wheat-maize-wheat-maize-wheat	Yes	Yes**	Ploughing	Yes
	CA6	Vertisol	Beef	30-40 t/ha	Maize-wheat-maize-wheat-maize	Yes	Yes**	Ploughing	No

* Soil type was determined according to pedological maps by Tanasijević et al. (1964) and Nejgebauer et al. (1971)
** Only herbicides used, no fungicides

Based on farmers' responses to the questionnaire (Table 5), it was observed that the quantity of manure amendments added to fields (where applicable) ranges from 14.5 t/ha at fields near Novi Karlovci, up to 80 t/ha at fields near Valjevo and Mionica. At all fields, mineral fertilizers were used, as well as pesticides. However, at fields from western/central Serbia (near Valjevo, Mionica and Čačak), only herbicides were used, without fungicides application. Management of postharvest residues is mainly ploughing at all locations, except at three fields near Valjevo, i.e., field VA2 (burning), and fields VA5 and VA6 (ploughing and burning). Finally, based on farmers' observations, at fields near Novi Karlovci, Valjevo and Mionica, there was no observed presence of wheat fusariosis in recent years, while at locations near Sombor and Čačak, there were fields with observed presence of wheat fusariosis.

5.2. Soil fungistasis to *Fusarium graminearum*

In the fungistasis experiment, the aim was to assess the impact of a biotic component on soil fungistasis to *F. graminearum*, by testing changes of *F. graminearum* abundance in autoclaved and non-autoclaved soils. Results showed that before soil inoculation (day 0), as well as in non-autoclaved, non-inoculated soils (day 15), *F. graminearum* Fg1 was not found in any of the 26 soils analyzed. When inoculated, autoclaved soils were used, growth of *F. graminearum* Fg1 took place in all soils during the 15 days of soil incubation, to a magnitude of 2 $\log_{10}$ units or more (Figure 8A). When inoculated, non-autoclaved soils were used, levels of *F. graminearum* Fg1 were always lower than with autoclaved soils. The pathogen levels were stable, or even increased in 16 of 26 non-autoclaved soils, while, interestingly, the amount of Fg1 DNA decreased in the remaining 10 (i.e., 38%; soils MI3, MI2, VA7, VA5, VA4, VA2, CA6, CA4, VA1 and CA3, all from western/central Serbia), indicating a fungistasis potential.

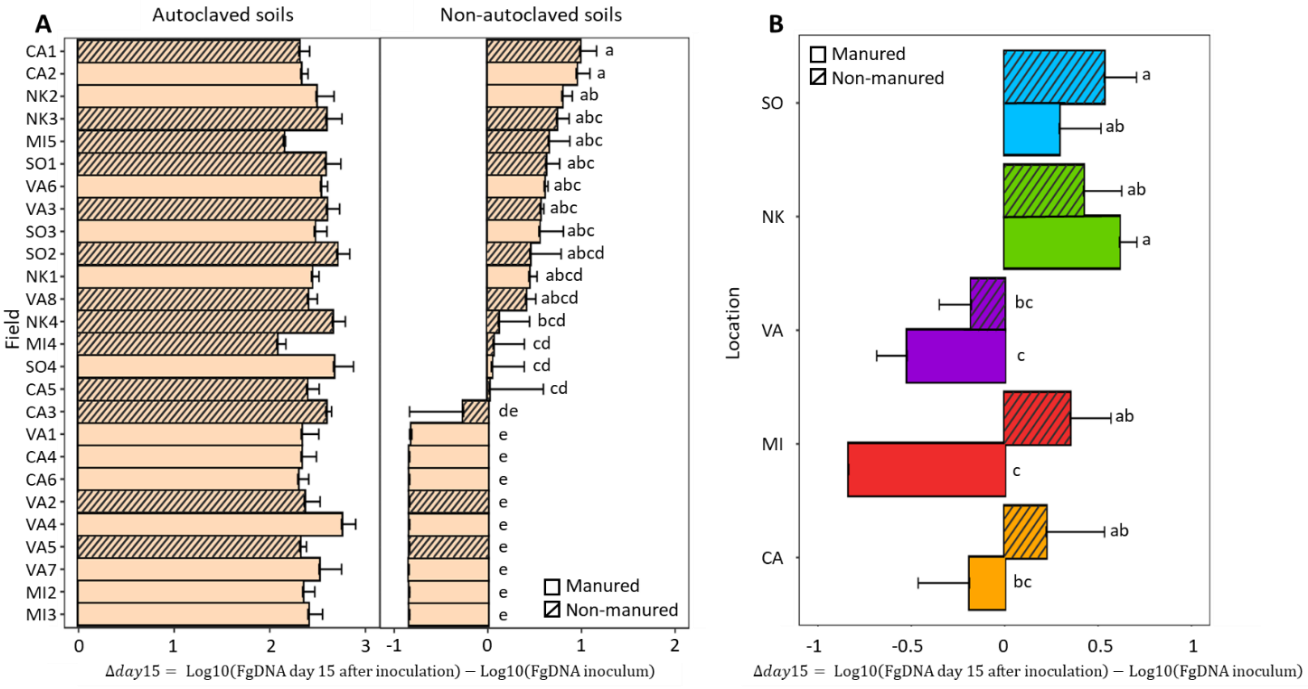

Figure 8. Amount of *Fusarium graminearum* Fg1 DNA present in the soils after 15 days of incubation. (A) DNA quantity of *Fusarium graminearum* Fg1 in inoculated 26 Serbian soils, from Valjevo (VA), Sombor (SO), Novi Karlovci (NK), Mionica (MI) and Čačak (CA), in autoclaved and

non-autoclaved soils, after 15 days of incubation under controlled conditions. Results are presented as means with standard errors. Striped bars indicate soils without manure amendments and non-striped bars indicate soils with manure amendments. Differences between individual soils were assessed with ANOVA and LSD tests ($P < 0.05$; letters a-e are used to show statistical differences). (B) Comparison of manured vs. non manured soils at each location. Results are presented as means with standard errors. Striped bars are used for soils without manure amendments and non-striped bars for soils with manure amendments. Differences between manured vs. non-manured soils at all locations were tested with ANOVA and LSD tests ($P < 0.05$; letters a-c are used to show statistical differences).

Two-way ANOVA ($P < 0.05$) showed that field location and manure amendments were significant factors, but the interaction between them was not significant. When considering manure amendments, seven of 10 non-autoclaved soils (70%) displaying fungistasis had been amended, whereas only six of 16 non-autoclaved, non-fungistatic soils (37%) had received manure (Figure 8A). When locations were compared (Figure 8A), fungistasis was found for the three western/central Serbia locations (Valjevo, Mionica, and Čačak) for manure-amended soils, but only for Valjevo and Čačak for non-manured soils. This global relationship between manure amendments and fungistasis was not significant at $P < 0.05$ ($Chi^2 = 1.463$), but comparison of all manured and non-manured soils from each location showed that manure amendment was associated with fungistasis in soils from Mionica ($P < 0.01$), with a similar trend (but $P > 0.05$) in soils from Čačak (Figure 8B).

In summary, fungistasis was observed for 38% of the 26 soils, and manure amendment was identified as a factor determining fungistasis in some (especially Mionica), but not all geographic locations. Considering the impact of manure amendments at location near Mionica (soils MI2, MI3, MI4 and MI5), these soils were re-sampled and *in planta* suppressiveness assay was performed in the next phase of research.

5.3. Suppressiveness of soils from Mionica against *Fusarium graminearum* - induced wheat damping-off

Based on the contrasting fungistasis results and the link with manure amendments found at Mionica, these soils (i.e., soils MI4 and MI5 were non-manured and non-fungistatic, while soils MI2 and MI3 were manured and fungistatic) were selected for a wheat damping-off suppressiveness assay with *F. graminearum* Fg1. At 14 days after sowing, the number of germinated seeds was statistically lower upon pathogen inoculation in soil MI4, whereas the difference was not significant in soils MI2, MI3, and MI5 (Figure 9A). Similarly, at four weeks, the number of plants alive was statistically lower in *F. graminearum* Fg1-inoculated vs. non-inoculated MI4 soils, while the difference was not significant in the three other soils (Figure 9B).

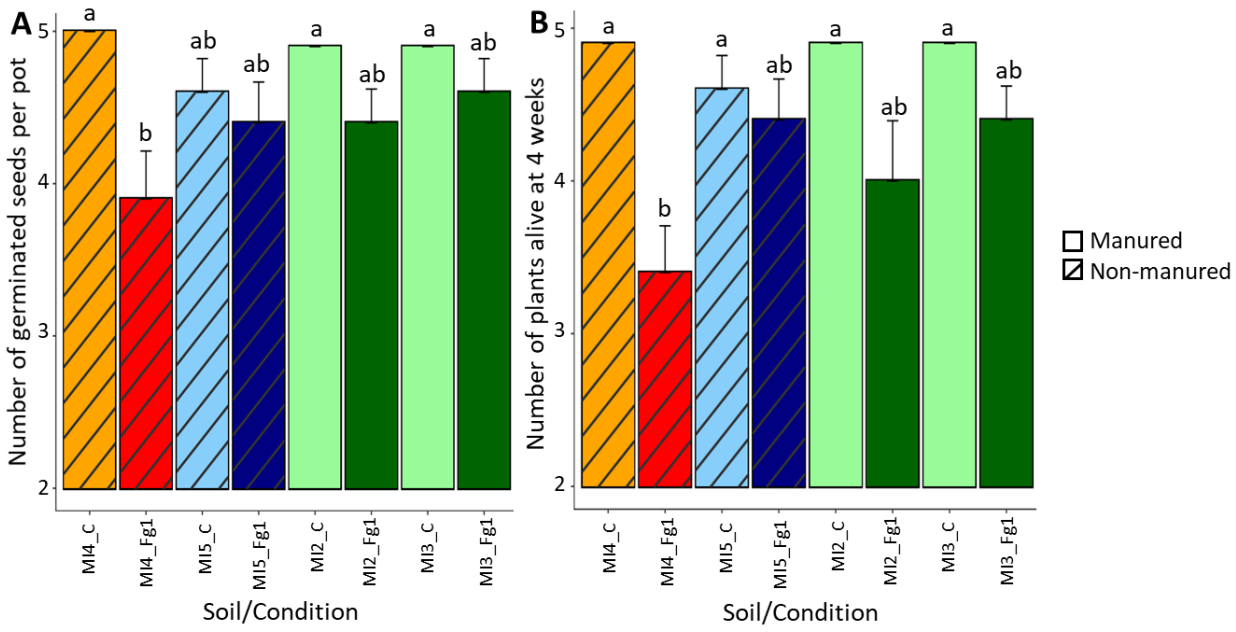

Figure 9. Wheat suppressiveness assay with MI4, MI5, MI2 and MI3 soils, non-inoculated (shown as MIi_C) or inoculated with *Fusarium graminearum* Fg1 (shown as MIi_Fg1). Soils that did not receive manure amendments are represented with stripes. Soils MI2 and MI3 are represented with the same colour, as they are both fungistatic and suppressive. All results are presented as means + standard errors (n = 10). Data were analyzed using Kruskal-Wallis and Dunn's tests ($P < 0.05$). For each soil, statistical differences are shown with letters a and b. (A) Number of germinated wheat seeds per pot (out of five) at two weeks. (B) Number of wheat plants alive per pot at four weeks.

Inoculation with *F. graminearum* Fg1 did not significantly impact wheat shoot length (Figure 10A), but it resulted in lower dry shoot biomass (Figure 10B) and shoot density (Figure 10C) in soil MI2. In addition, dry shoot biomass, shoot length and shoot density were higher overall (*t* tests, all $P < 10^{12}$) in manured soils (MI2 and MI3) than in non-manured soils (MI4 and MI5), when both *F. graminearum* Fg1-inoculated and non-inoculated soils were taken together.

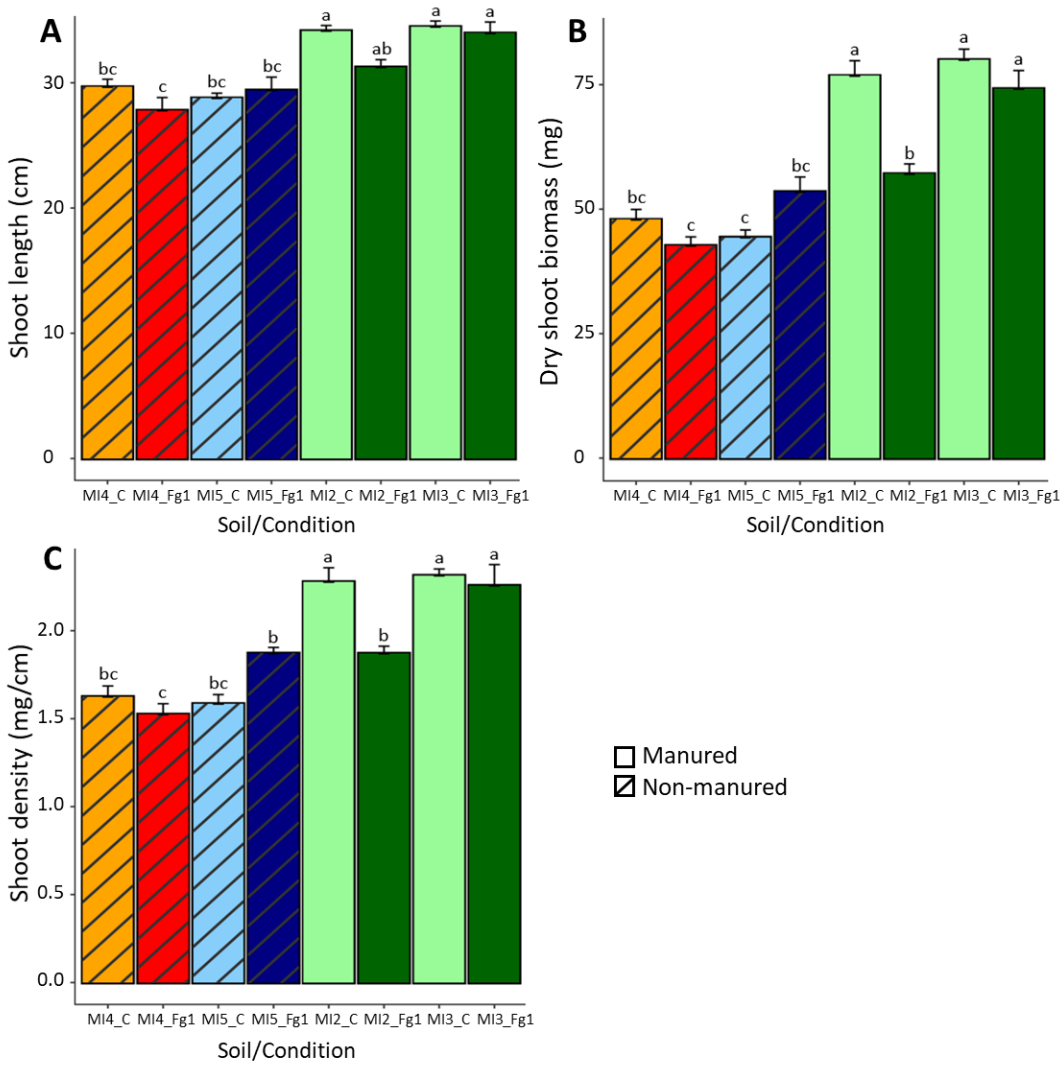

Figure 10. Wheat suppressiveness assay with MI4, MI5, MI2 and MI3 soils, non-inoculated (shown as MIi_C) or inoculated with *Fusarium graminearum* Fg1 (shown as MIi_Fg1). Soils that did not receive manure amendments are represented with stripes. Soils MI2 and MI3 are represented with the same colour, as they are both fungistatic and suppressive. All results were obtained at four weeks and are presented as means + standard errors (n = 10). Non-germinated plants were regarded as missing data (NA). Data were analyzed using ANOVA and Tukey's HSD test ($P < 0.05$). Statistical differences are shown with letters a to c. (A) Shoot length (cm). (B) Dry shoot biomass (mg). (C) Shoot density (mg/cm).

In summary, based on fungistasis and suppressiveness assays, three soil categories were observed:

(i) Soil MI4 (non-manured) was non-fungistatic and also non-suppressive,
(ii) Soil MI5 (non-manured) was non-fungistatic but suppressive, whereas
(iii) Soils MI2 and MI3 (manured) were fungistatic and suppressive to wheat damping-off caused by *F. graminearum* Fg1.

Based on these results, one soil from each of the three categories was chosen, i.e., soil MI4 (non-fungistatic and non-suppressive), MI5 (non-fungistatic and suppressive) and MI2 (fungistatic and suppressive) to perform the metabarcoding analysis of prokaryotic and fungal diversity, using the rhizospheres of wheat plants (both inoculated and non-inoculated with *F. graminearum* Fg1) grown during the suppressiveness assay. Between soils MI2 and MI3, soil MI2 was chosen at random. In both datasets, the rarefaction curves tended to reach a plateau, indicating that the sequencing method supplied sufficient sequences to cover most of the diversity (Supplementary material; Chapter 2; Figure S1).

5.4. Diversity of the prokaryotic and fungal rhizospheric communities in soils from Mionica

When assessing the link between rhizosphere microbial diversity and disease-suppressiveness status of soils from Mionica, metabarcoding data for the 16S rRNA gene (prokaryotic community) pointed to similar diversity levels for the three soils (Figure 11ABC). This was found whether soils were inoculated with *F. graminearum* Fg1 or not, except that Pielou index (a measure of species evenness) was significantly higher in the fungistatic, suppressive soil MI2 (also the only manured soil), than in non-fungistatic soils MI4 (conducive) and MI5 (suppressive), when inoculated with Fg1 (Figure 11C). Besides that, the effect of Fg1 inoculation on alpha diversity was not significant, regardless of the soil and the diversity index.

With ITS metabarcoding data (fungal community) from the rhizosphere, the Shannon (species diversity; Figure 11D) and Pielou (species evenness; Figure 11F) indices were statistically higher (i) in soils MI4 (non-fungistatic, non-suppressive) and MI2 (fungistatic, suppressive) than in MI5 (non-fungistatic, suppressive) in the absence of inoculation, and (ii) in soil MI4 than in MI5 when *F. graminearum* Fg1 had been inoculated. Inoculation itself resulted only in a lower Pielou index in soil MI4 (Figure 11F). There were no statistical differences in the Chao1 index (species richness; Figure 11E) between different soils and inoculation conditions.

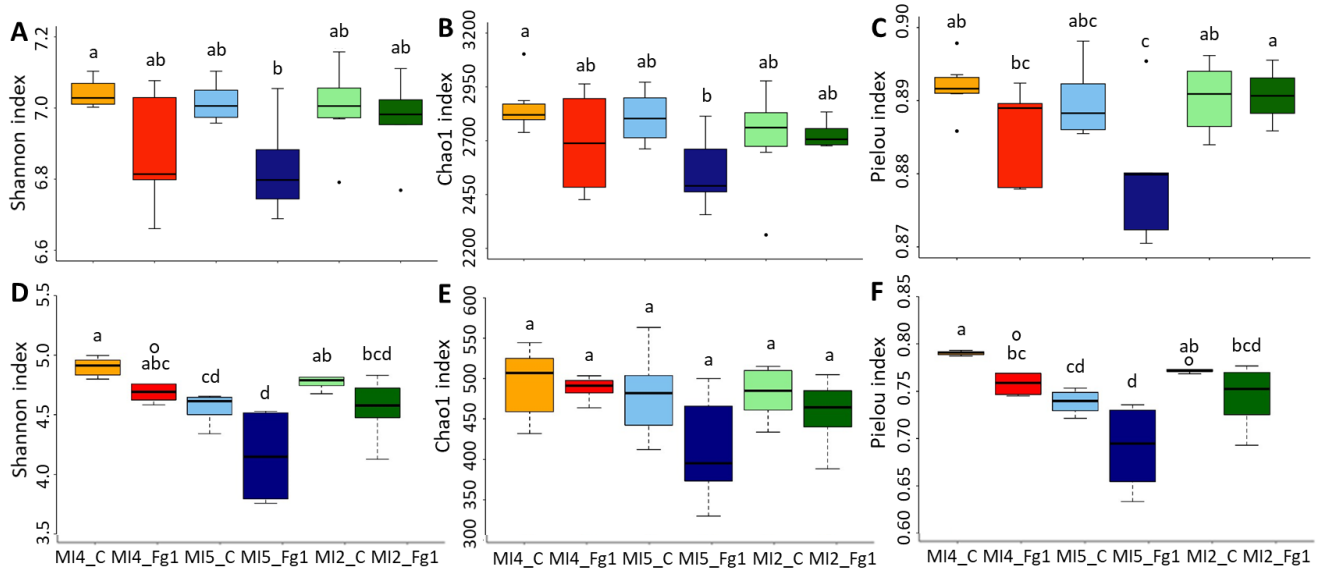

Figure 11. Alpha diversity of prokaryotic (A, B, C) and fungal (D, E, F) rhizosphere communities in soils MI4 (non-fungistatic and non-suppressive), MI5 (non-fungistatic and suppressive) and MI2 (fungistatic and suppressive), inoculated with *Fusarium graminearum* Fg1 (shown as

MIi_Fg1) or non-inoculated (shown as MIi_C). Data were compared using Kruskal–Wallis tests, followed by Fisher's Least Significant Difference (LSD) tests with Bonferroni correction. Letters a-d indicate statistical relations ($P < 0.05$) between soils × inoculation (*Fusarium graminearum* Fg1 or not) combinations.

NMDS plots based on Bray-Curtis distances showed that microbial communities are clustered largely according to the field of origin, for the prokaryotic (Figure 12A) and especially the fungal community (Figure 12B). ANOSIM (10,000 permutations) indicated that the between-groups difference was larger than the within-groups difference ($P = 10^{-4}$ for prokaryotes and 10^{-4} for fungi). All pairwise comparisons (for MI5 vs. MI4, MI5 vs. MI2, and MI4 vs. MI2) for prokaryotes were $P = 10^{-3}$ and $P = 10^{-3}$ for fungi.

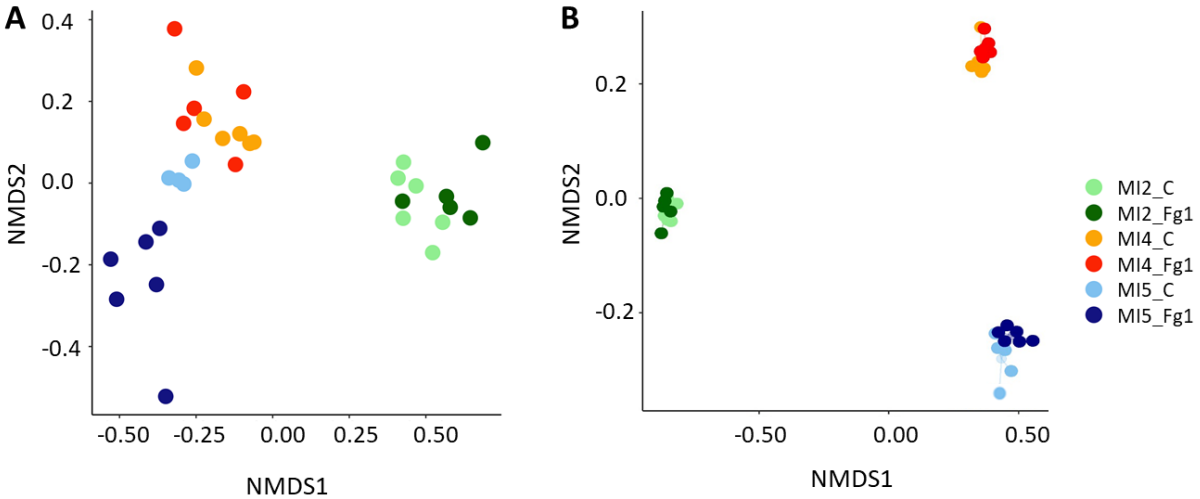

Figure 12. Non-Metric Multidimensional Scaling (NMDS) of soils MI4 (non-fungistatic and non-suppressive), MI5 (non-fungistatic and suppressive) and MI2 (fungistatic and suppressive), inoculated with *Fusarium graminearum* Fg1 (shown as MIi_Fg1) or non-inoculated (shown as MIi_C) based on rhizosphere metabarcoding of prokaryotic (A) and fungal (B) communities. The shorter the distance between the samples (dots), the greater the similarity between the microbial communities.

Indeed, PERMANOVA results indicated that individual soils accounted for 42.6% (for prokaryotes) and 60.0% (for fungi) of the variations in community structure (both at $P < 0.001$), whereas merely 3.7% (prokaryotes; $P = 0.048$) and 4.0% (fungi; $P = 0.023$) of the differences were attributed to the inoculation (Table 6). When considering each soil separately, the effect of *F. graminearum* Fg1 inoculation was significant in most cases, specifically for MI4 ($P = 0.004$ for prokaryotes but $P > 0.05$ for fungi), MI5 ($P = 0.004$ for prokaryotes and $P = 0.048$ for fungi) and MI2 ($P = 0.009$ for prokaryotes and $P = 0.004$ for fungi).

Table 6. PERMANOVA performed on (A) 16S rRNA and (B) ITS Illumina MiSeq datasets, based on Bray-Curtis distances, with 10^4 permutations. Results are presented for all soils and conditions together, and for each soil separately.

A. All treatments				**B.** All treatments			
Source of variation	R^2	F	*P* value	Source of variation	R^2	F	*P* value
Soil	0.42	14.87	< 0.001	Soil	0.6	24.91	< 0.001
Inoculation	0.03	2.26	0.048	Inoculation	0.04	3.3	0.023
Soil × Inoculation	0.05	1.53	0.11	Soil × Inoculation	0.03	1.1	0.316
MI4				MI4			
Source of variation	R^2	F	*P* value	Source of variation	R^2	F	*P* value
Inoculation	0.14	1.53	0.004	Inoculation	0.14	0.63	0.065
MI5				MI5			
Source of variation	R^2	F	*P* value	Source of variation	R^2	F	*P* value
Inoculation	0.21	2.23	0.004	Inoculation	0.17	1.9	0.048
MI2				MI2			
Source of variation	R^2	F	*P* value	Source of variation	R^2	F	*P* value
Inoculation	0.15	1.67	0.009	Inoculation	0.18	2	0.004

In summary, most differences in prokaryotic alpha diversity were not significant, whereas fungi in soil MI5 (non-fungistatic, suppressive) displayed lower Shannon and Pielou indices. In addition, microbial community structure depended mostly on the field of origin, with a modest, but significant, effect of inoculation.

5.5. Taxonomic rhizosphere community composition in soils from Mionica

5.5.1. Taxonomic composition of the prokaryotic rhizosphere community in soils from Mionica

Taxonomic composition of the prokaryotic community in soils from Mionica showed that the most abundant rhizosphere phyla in soils MI4, MI5 and MI2 were the same, i.e., *Proteobacteria*, *Actinobacteriota, Firmicutes, Chloroflexi, Verrucomicrobiota* and *Crenarchaeota.* The 20 most abundant taxa (the lowest likely taxonomic information available for an ASV, often at the genus level) in the prokaryotic community represented 53.2% (for non-inoculated MI4 soil), 57.7% (for *F. graminearum* Fg1-inoculated MI4 soil), 55.7% (for non-inoculated MI5 soil), 60.5% (for *F. graminearum* Fg1-inoculated MI5 soil), 55.3% (for non-inoculated MI2 soil) and 53.9% (for *F. graminearum* Fg1-inoculated MI2 soil) of the reads in rhizosphere samples (Figure 13ABC). Some of these most abundant taxa were evidenced in all three soils, e.g., the *Actinobacteriota*, *Gaiella*

and a taxon affiliated to the order *Gaiellales*. Some were found in specific soil(s), as for (i) the *Proteobacteria* genus *Sphingomonas* in soil MI4, (ii) an *Elsterales* (*Proteobacteria*) genus in soil MI5, (iii) an *Acidobacteriota* taxon from the order *Vicinamibacterales* and various *Actinobacteriota*, i.e., the genera *Microlunatus* and *Rubrobacter*, a *Microtrichales* genus and a *Ilumatobacteraceae* genus, which were evidenced only in soil MI2, and (iv) the *Actinobacteriota* genera *Conexibacter, Marmoricola*, *Intransporangium* and *Acidothermus* in soils MI4 and MI5.

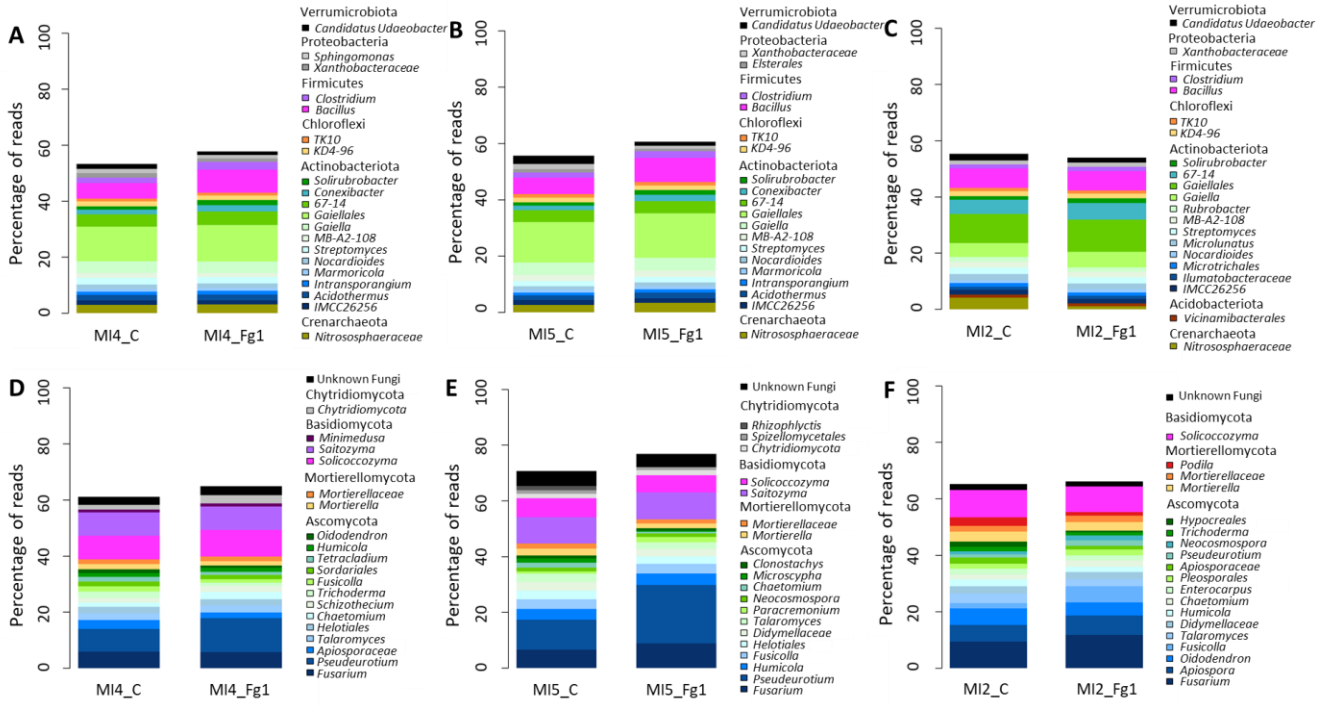

Figure 13. Top 20 most abundant prokaryotic (A, B, C) and fungal taxa (D, E, F) in the wheat rhizosphere of soils from Mionica, MI4, MI5 and MI2. MI_C, control (non-inoculated soils); MIi_Fg1, *Fusarium graminearum* Fg1-inoculated soils.

As for the prokaryotic community and impact of inoculation with *F. graminearum* Fg1, it resulted in a significant increase ($P < 0.05$) in the rhizosphere relative abundance of the phylum *Firmicutes* in the non-fungistatic soils MI4 (from 10.8% to 15.8%) and MI5 (from 10.1% to 14.4%) (Figure 14AB). In the fungistatic MI2 soil, pathogen inoculation caused a modest but significant increase ($P < 0.05$) in the relative abundance of *Actinobacteriota* (from 48.0% to 50.9%) and *Proteobacteria* (from 13.7% to 17.1%), but led to somewhat lower levels of *Crenarchaeota* (from 5.4% to 1.5%) and *Chloroflexi* (from 5.4% to 4.9%) ($P < 0.05$) (Figure 14C).

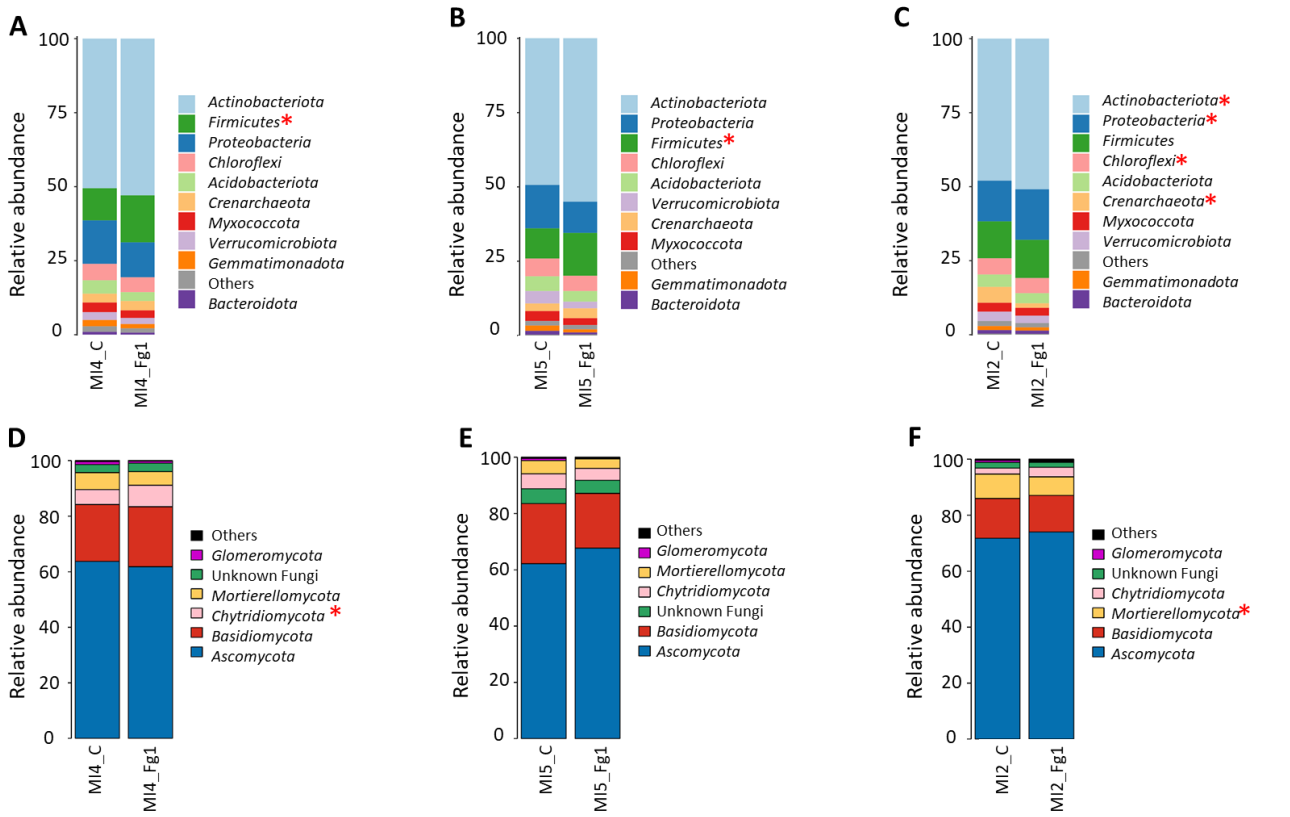

Figure 14. Relative abundance of prokaryotic (A, B, C) and fungal phyla (D, E, F) in the rhizosphere of soils from Mionica, MI4, MI5 and MI2. MIi_C, control (non-inoculated soils); MIi_Fg1, *Fusarium graminearum* Fg1-inoculated soils. Asterisks indicate significant differences in the relative abundance of phyla in each inoculated vs. non-inoculated soil based on Kruskal-Wallis tests followed by Fisher's Least Significant Difference (LSD) tests with Bonferroni correction ($P < 0.05$).

Subsequently, differential analysis was also used to identify individual taxa that differed significantly ($P < 0.05$) in relative abundance between *F. graminearum* Fg1-inoculated and non-inoculated samples, at the scale of the whole prokaryotic rhizosphere community. Among the 1493 identified prokaryotic taxa, this concerned 17 taxa in soil MI4 (non-fungistatic, non-suppressive), 45 taxa in soil MI5 (non-fungistatic, suppressive), and 17 taxa in soil MI2 (fungistatic, suppressive) (Figure 15). Most of the taxa were found exclusively in one of the three soils, but *Gemmatimonas* (*Gemmatimonadota*) was evidenced in all three soils, with a lower abundance in inoculated than in non-inoculated samples (by 0.5, 1.2, and 0.8 $\log_2$ units for soils MI4, MI5 and MI2, respectively). In both soils MI4 and MI5, a taxon belonging to the candidate group SC-I-84 (*Proteobacteria*) was present more abundantly in non-inoculated than in inoculated samples (by 0.8 and 1.2 $\log_2$ units, respectively), as was a *Myxococcota* taxon from the candidate group Blrii41 (by 0.6 and 1.2 $\log_2$ units, respectively), whereas the opposite was detected for *Bacillus* (by 0.7 and 0.9 $\log_2$ units, respectively), *Paenibacillus* (by 0.8 and 0.9 $\log_2$ units, respectively) and *Pelosinus* (*Firmicutes*) (by 1.2 and 2.0 $\log_2$ units, respectively). *Sphingobium* (*Proteobacteria*) was more abundant in inoculated than in non-inoculated samples of soils MI2 and MI5 (by 7.0 and 8.0 $\log_2$ units, respectively). These inoculation effects were also evident for some of the 20 most abundant prokaryotic taxa, such as *Solirubrobacter* in soil MI4, *Candidatus Udaeobacter* and *Bacillus* in soil MI5 and *Nitrososphaeraceae* in soil MI2.

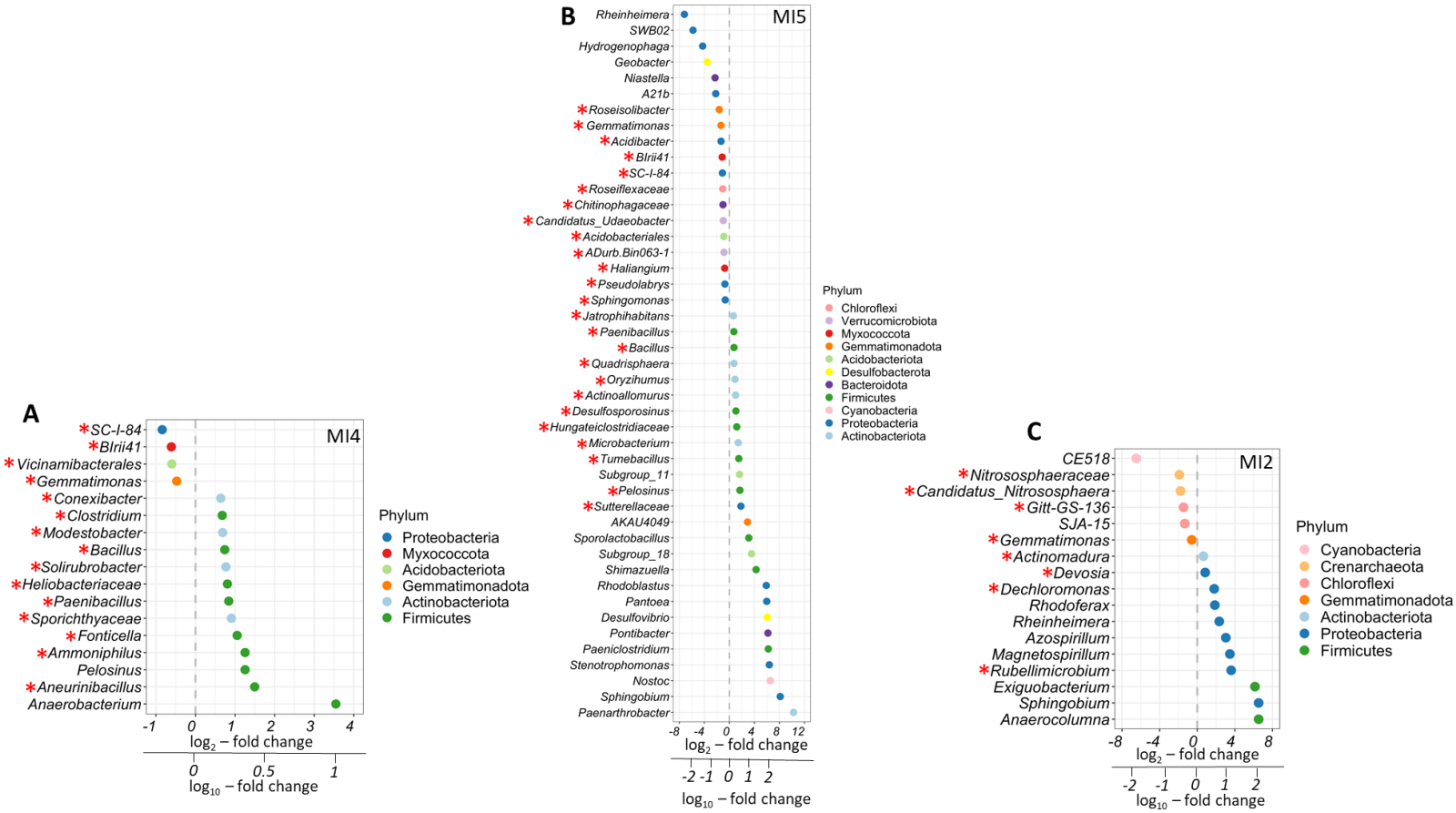

Figure 15. Differential abundance analysis of prokaryotic taxa in the wheat rhizosphere of soils MI4 (A), MI5 (B) and MI2 (C) following inoculation with *Fusarium graminearum* Fg1. The X axes are shown with $\log_2$ and $\log_{10}$ changes. Negative log changes (significantly more abundant in non-inoculated soils); positive log changes (significantly more abundant in *Fusarium graminearum* Fg1-inoculated soils). All taxa shown were affected by inoculation ($P < 0.05$), and those representing more than 0.1 % of all the sequences are indicated with an asterisk.

In summary, the wheat rhizosphere of the three soils shared the main prokaryotic phyla and the majority of the most abundant taxa, although several taxa were soil-specific. Additionally, soil inoculation with *F. graminearum* Fg1 impacted the rhizosphere microbial community, but often with soil-specific effects.

5.5.2. Taxonomic composition of the fungal rhizosphere community in soils from Mionica

As for the fungal community in soils from Mionica, in each soil *Ascomycota*, *Basidiomycota*, and *Mortierellomycota* were the phyla harboring the most abundant taxa. However, differences were found between soils, as in soils MI4 and MI5 the phylum *Chytridiomycota* was also present. The 20 most abundant fungal taxa (considered at the genus level or higher rank) represented 61.1% (for non-inoculated MI4 soil), 64.8% (for *F. graminearum* Fg1-inoculated MI4 soil), 70.7% (for non-inoculated MI5 soil), 76.9% (for *F. graminearum* Fg1-inoculated MI5 soil), 65.2% (for non-inoculated MI2 soil), and 66.1% (for *F. graminearum* Fg1-inoculated MI2 soil) of the reads in rhizosphere samples (Figure 13DEF). Distinctive features were evidenced in particular soil(s), as (i) *Schizothecium*, *Sordariales*, *Tetracladium* and *Minimedusa* were found only in soil MI4, (ii) *Clonostachys*, *Microscypha* and *Paracremonium* only in soil MI5, (iii) *Podila* (a *Mortierellaceae* genus; representing 10% of the reads), *Hypocreales*, *Apiospora*, *Pleosporales* and *Enterocarpus* were found only in soil MI2, (iv) *Pseudeurotium, Helotiales, Humicola* and *Saitozyma* only in soils MI4 and MI5, (v) *Apiosporaceae*, *Chaetomium*, *Trichoderma* and *Oidodendron* only in soils MI4 and MI2, and (vi) *Neocosmospora* and *Didymellaceae* only in soils MI5 and MI2.

At the phylum level, inoculation with *F. graminearum* Fg1 resulted into a significant increase ($P < 0.05$) in the rhizosphere relative abundance of the *Chytridiomycota* in the non-fungistatic soil MI4 (from 5.4% to 7.8%) (Figure 14D), and a decrease of the *Mortierellomycota* (from 8.7% to 6.6%) in the fungistatic soil MI2 (Figure 14F), whereas there were no observed differences in the relative abundance of any fungal phyla in the soil MI5 upon pathogen inoculation (Figure 14E).

Similarly as with the prokaryotic community, when differential analysis was performed to assess inoculation effects at the scale of the whole fungal community (Figure 16), decreased levels were found in *F. graminearum* Fg1-inoculated soils for: (i) *Ascomycota* genera *Beauvaria* (by 5.8 $\log_2$ units) and *Collarina* (by 4.9 $\log_2$ units) and *Mortierellomycota* genus *Podila* (by 1.8 $\log_2$ units) in soil MI4 (non-fungistatic, non-suppressive), for (ii) four genera (of distinct phyla) including *Waitea* (*Basidiomycota*; by 24 $\log_2$ units), *Microscypha* (*Ascomycota*; by 2 $\log_2$ units), *Paraglomus* (*Glomeromycota*; by 7 $\log_2$ units) and *Rhizophlyctis* (*Chytridiomycota*; by 4 $\log_2$ units) in soil MI5 (non-fungistatic, suppressive), and (iii) the four *Ascomycota* genera *Septoria* (by 23 $\log_2$ units), *Purpureocillium* (by 2 $\log_2$ units), *Scedosporium* (by 6 $\log_2$ units) and *Exophiala* (by 2.5 $\log_2$ units) in soil MI2 (fungistatic, suppressive). Significantly higher levels were found in *F. graminearum* Fg1-inoculated MI2 soil for *Atractium* (by 2.5 $\log_2$ units) and *Scutellinia* (by 3.0 $\log_2$ units). Inoculation effects were also observed (Kruskal-Wallis tests and Fisher's tests with Bonferroni correction) for some of the 20 most abundant fungal taxa, but these effects were at $P > 0.05$, when the differential analysis was employed.

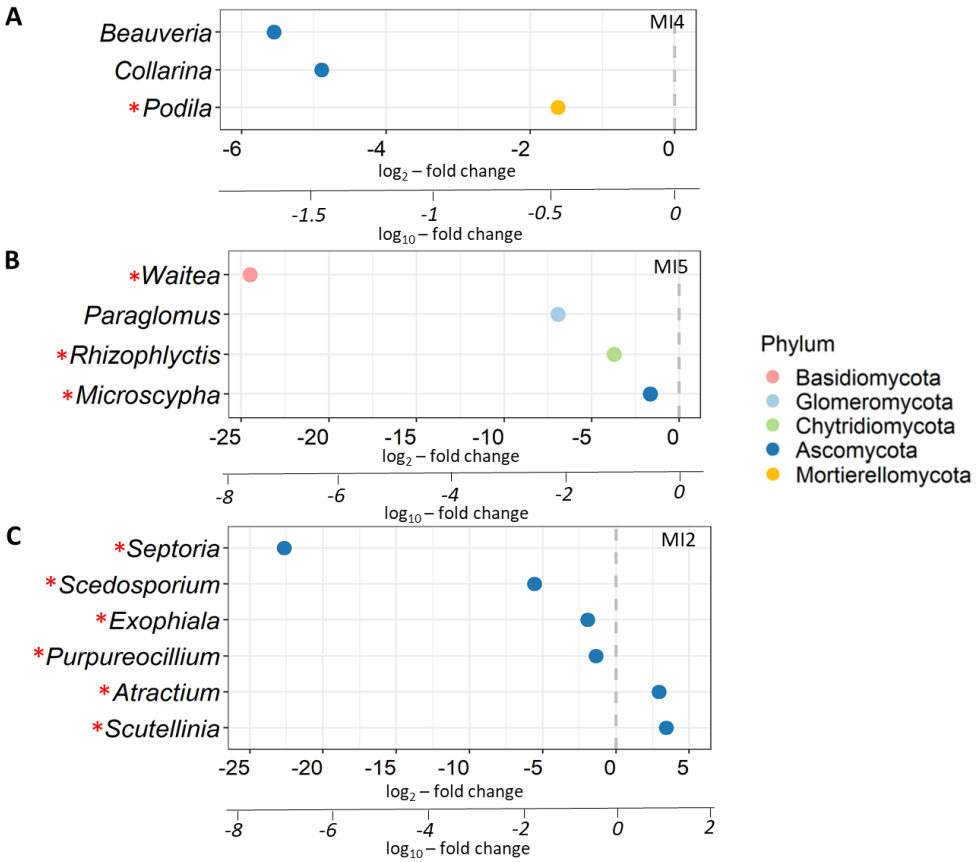

Figure 16. Differential abundance analysis of fungal taxa in the wheat rhizosphere of soils MI4 (A), MI5 (B) and MI2 (C) following inoculation with *Fusarium graminearum* Fg1. The X axes are shown with $\log_2$ and $\log_{10}$ changes. Negative log changes (significantly more abundant in non-inoculated soils); positive log changes (significantly more abundant in *Fusarium graminearum* Fg1-inoculated soils). All taxa shown were affected by inoculation ($P < 0.05$), and those representing more than 0.1 % of all sequences are indicated with an asterisk.

In summary, for the fungal community, the three soils harbored representatives from the phyla *Ascomycota*, *Basidiomycota*, and *Mortierellomycota*, while taxa from the order *Chytridiomycota* were found only in soils MI4 and MI5. For the fungal community, soil inoculation with *F. graminearum* Fg1 impacted the rhizosphere community.

5.5.3. Composition of the *Fusarium* community in soils from Mionica

After analyzing diversity and taxonomic composition of prokaryotic and fungal community of soils from Mionica MI4, MI5 and MI2, the composition of *Fusarium* populations in these soils (both inoculated and non-inoculated with *F. graminearum* Fg1) was also assessed. It was shown that in the absence of *F. graminearum* inoculation, the *Fusarium* genus represented 9.4% of all rhizosphere fungi in non-suppressive soil MI4 (also non-fungistatic), vs. only 5.9% and 6.6% in suppressive soils MI5 (non-fungistatic) and MI2 (fungistatic), respectively (Table 7). The *Fusarium* genus was more prevalent in Fg1-inoculated vs. non-inoculated rhizosphere for soil MI4 (up to 11.8%) and MI2 (up to 8.8%), but not for soil MI5.

Table 7. Relative abundance of the *Fusarium* genus among fungi and of individual *Fusarium* species among the *Fusarium* genus. MIi_C, non-inoculated soils; MIi_Fg1, *Fusarium graminearum* Fg1-inoculated soils. Asterisks indicate significant difference in the relative abundance of individual *Fusarium* species in inoculated vs. non-inoculated soil based on Kruskal-Wallis tests followed by Fisher's Least Significant Difference (LSD) tests with Bonferroni correction ($P < 0.05$).

Soil/ inoculation	*F. burgessii*	*F. croci*	*F. equiseti*	*F. graminearum*	*F. hostae*	*F. incarnatum*	*F. neocosmosporiellum*	*F. nygamai*	*F. oxysporum*	*F. tricinctum*	*Fusarium* unclassified	% *Fusarium* among total fungal reads
MI4_C	0.00	0.00	2.28	2.63	6.29	0.11	3.75	0.57	76.98	0.71	6.68	9.41
MI4_Fg1	0.03	0.00	1.45*	25.48*	5.40	1.19	2.58	0.54	57.09	1.16	5.08	11.82
MI5_C	0.04	0.00	0.86	0.94	10.23	0.00	2.99	0.31	78.02	2.35	4.27	5.93
MI5_Fg1	0.00	0.00	0.60	29.36*	5.54	0.01	2.09	0.00	58.81	0.24	3.37	5.71
MI2_C	0.00	0.00	19.26	0.53	0.26	0.00	1.87	0.23	66.96	3.31	7.58	6.59
MI2_Fg1	0.00	0.03	17.68	19.98*	0.07	0.00	1.78	0.14	54.07	1.81	4.45*	8.83

Within the genus, inoculation significantly increased ($P < 0.05$) rhizosphere levels of *F. graminearum* from 2.6% to 25.0% of all *Fusarium* sequences for MI4, 0.9% to 29.0% for MI5, and 0.5% to 20.0% for MI2 (Table 7; Figure 17). In addition, a small decrease in levels of *F. equiseti* was detected in soil MI4 after inoculation (Table 7; Figure 17).

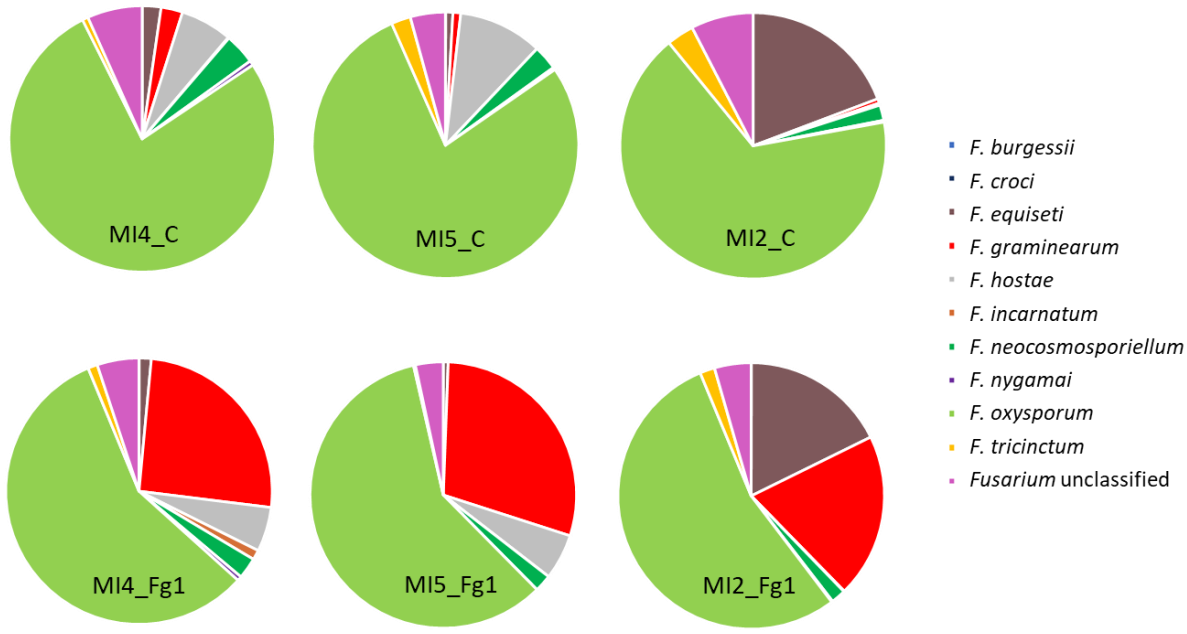

Figure 17. Proportion of the different *Fusarium* species in the wheat rhizosphere of soils MI4, MI5 and MI2 inoculated (MIi_Fg1) or not inoculated (MIi_C) with *Fusarium graminearum* Fg1.

All the raw amplicon data for 16S rRNA gene and ITS have been deposited into the NCBI Sequence Read Archive (SRA), under the BioProject PRJNA1010537.

5.6. Fungistatic soils as a source of rhizosphere bacteria with biocontrol properties against *Fusarium graminearum*

Aiming to assess the usefulness of fungistatic soils as a source of biocontrol agents against *F. graminearum*, isolation, characterization, genome sequencing and *in planta* assay of diverse taxa from both fungistatic and non-fungistatic soils, were performed.

5.6.1. Antagonistic activity of rhizosphere bacteria against *Fusarium graminearum* Fg1

In this phase of research, 244 bacteria of contrasted taxonomy were isolated from the rhizospheres of wheat plants grown in MI (near Mionica) or CA (near Čačak) soils – specifically, 118 from fungistatic soils (MI2, MI3 and CA3) and 126 from non-fungistatic soils (MI4, MI5, CA1 and CA2) (Table 8), and subjected to dual culture test with *F. graminearum*. Soils MI and CA have been chosen for bacterial isolation, based on the following criteria:

(i) Both of these soils are of the same soil type-vertisol (Table 5), and
(ii) At both of these locations, there are soils from fields that are fungistatic and non-fungistatic towards *F. graminearum* Fg1 (Figure 8AB).

Table 8. Number of isolates obtained from each fungistatic soil (MI2, MI3 and CA3) and non-fungistatic soil (MI4, MI5, CA1 and CA2), with the corresponding isolation media. NA: Nutrient agar; NAsp: Nutrient agar plated with pasteurized soil sample, aiming to isolate sporogene bacteria; TSA: Tryptone soya agar; KB: King's B; C: Cetrimide agar; F: Fiodorov agar; SAA: Starch ammonia agar.

Soil	Total number of isolates							
	NA	NAsp	TSA	KB	C	F	SAA	Total
Fungistatic soils								
MI2	12	8	10	8	3	5	6	**52**
MI3	11	8	9	3	5	4	2	**42**
CA3	6	5	2	4	5		2	**24**
Non-fungistatic soils								
MI4	7	6	8	8	4	5	3	**41**
MI5	10	7	8	9	2	4	1	**41**
CA1	7	4	6	2	4		5	**28**
CA2	2	3	4	2	5			**16**
Total	55	41	47	36	28	18	19	**244**

Tests based on their ability to inhibit mycelial growth for more than 50% or alter colony morphology of *F. graminearum* Fg1 *in vitro* resulted in the selection of 12 and 11 antagonistic isolates (none exhibiting both effects), respectively, accounting to a total of 23 isolates (9.4%) (Table 9). These 23 antagonistic isolates included 10 isolates from fungistatic soils and 13 from non-fungistatic soils, and 13 of the 23 originated from MI soils (six from the fungistatic and suppressive soils MI2, MI3 and four from the non-fungistatic and suppressive soil MI5, versus three from the non-fungistatic and non-suppressive soil MI4), while 10 originated from CA soils.

Table 9. Isolates from each fungistatic and non-fungistatic soil with the ability to inhibit *Fusarium graminearum* Fg1 mycelial growth (indicated with number; %) or alter colony morphology (indicated wth +), with the corresponding isolation media. Taxonomic affiliation of isolates based on 16S rRNA is also indicated. NA: Nutrient agar; NAsp: Nutrient agar plated with pasteurized soil sample, aiming to isolate sporogene bacteria; TSA: Tryptone soya agar; KB: King's B; C: Cetrimide agar; F: Fiodorov agar; SAA: Starch ammonia agar.

Soil	Isolate name	Isolation media	16S rRNA taxonomic identification	Antagonistic activity
Fungistatic soils				
MI2	IT-210MI2	NAsp	*Priestia* sp.	+
	IT-79MI2	TSA	*Bacillus* sp.	83
MI3	IT-74MI3	NAsp	*Bacillus* sp.	+
	IT-91MI3	TSA	*Kosakonia* sp.	+
	IT-180MI3	TSA	*Priestia* sp.	+
	IT-162MI3	C	*Pseudomonas* sp.	70
CA3	IT-19CA3	NA	*Bacillus* sp.	53
	IT-40CA3	KB	*Bacillus* sp.	68
	IT-51CA3	C	*Pseudomonas* sp.	68
	IT-53CA3	C	*Pseudomonas* sp.	+

Non-fungistatic soils				
MI4	IT-93MI4	KB	*Pseudomonas* sp.	+
	IT-194MI4	NA	*Pseudomonas* sp.	+
	IT-158MI4	C	*Burkholderia* sp.	70
MI5	IT-111MI5	KB	*Burkholderia* sp.	68
	IT-196MI5	NA	*Pseudomonas* sp.	+
	IT-232MI5	C	*Pseudomonas* sp.	63
	IT-133MI5	TSA	*Bacillus* sp.	75
CA1	IT-13CA1	NAsp	*Bacillus* sp.	59
	IT-43CA1	C	*Pseudomonas* sp.	+
CA2	IT-7CA2	NAsp	*Brevibacillus* sp.	95
	IT-36CA2	KB	*Chryseobacterium* sp.	+
	IT-47CA2	C	*Pseudomonas* sp.	+
	IT-48CA2	C	*Pseudomonas* sp.	75

Taxonomic affiliation of antagonistic isolates based on 16S rRNA sequences affiliated them to the genera *Bacillus* (six isolates), *Priestia* (formerly *Bacillus*; two isolates), *Pseudomonas* (10 isolates), *Kosakonia* (one isolate), *Burkholderia* (two isolates), *Brevibacillus* (one isolate) and *Chryseobacterium* (one isolate) (Table 9). Isolates belonging to genera *Pseudomonas*, *Bacillus*, *Burkholderia* and *Brevibacillus* inhibited growth of *F. graminearum* Fg1 from 53 to 95 %, while isolates belonging to genera *Priestia*, *Bacillus*, *Kosakonia*, *Pseudomonas* and *Chryseobacterium* had the ability to alter fungal colony morphology. For example, isolate *Brevibacillus* sp. IT-7CA2 inhibited the growth of *F. graminearum* Fg1 by 95%, and isolate *Chryseobacterium* sp. IT-36CA2 altered fungal sporulation (Figure 18AB). In summary, antagonistic bacterial isolates were obtained in similar numbers from fungistatic and non-fungistatic soils.

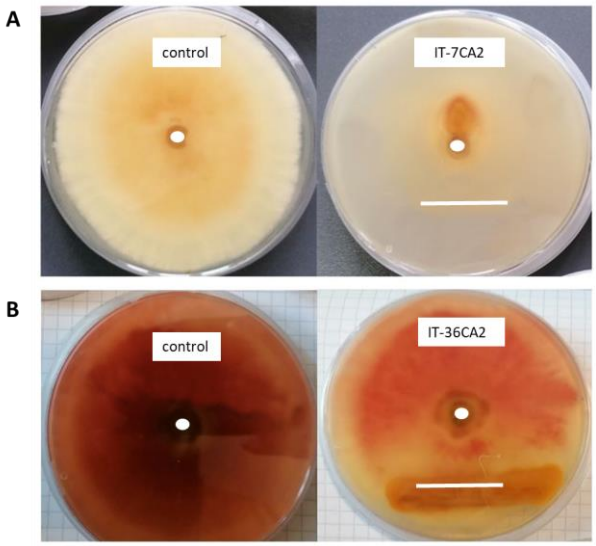

Figure 18. Antagonistic activity of bacterial isolates *Brevibacillus* sp. IT-7CA2 (A) and *Chryseobacterium* sp. IT-36CA2 (B) towards *Fusarium graminearum* Fg1 in a dual-culture assay. Left: control plate with *Fusarium graminearum* Fg1. Right: plate with *Fusarium graminearum* Fg1 and the bacterial isolate. The white dot on Petri dishes represents the place of inoculation of *Fusarium graminearum* Fg1 and the white line represents the point of bacterial inoculation.

In summary, out of 244 isolates obtained from both fungistatic (MI2, MI3 and CA3) and non-fungistatic (MI4, MI5, CA1 and CA2) soils, 23 had antagonistic activity against *F. graminearum* Fg1, and either inhibited fungal mycelial growth or altered fungal colony morphology. These 23 isolates belonged to the genera *Bacillus*, *Priestia*, *Pseudomonas*, *Kosakonia*, *Burkholderia*, *Brevibacillus* and *Chryseobacterium*.

5.6.2. Identification of antagonistic bacterial isolates through genome sequencing

After the 23 bacterial isolates with antagonistic activity against *F. graminearum* Fg1 have been obtained, all of their genomes have been sequenced and assembled. Whole genome sequences (raw and assembled) are deposited into the EBI/EMBL database under the accession number PRJEB59762. All 23 genome-sequenced, antagonistic isolates taxonomically differed from one another and they were distributed across three phyla and seven genera (Table 10). The 10 strains from fungistatic soils belonged to the phyla *Pseudomonadota* (formerly *Proteobacteria*) (three strains from the genus *Pseudomonas* and one from the genus *Kosakonia*) or *Bacillota* (formerly *Firmicutes*) (four strains from the genus *Bacillus* and two from the genus *Priestia*). The 13 strains from non-fungistatic soils belonged to the phyla *Pseudomonadota* (seven from *Pseudomonas* and two from *Burkholderia*), *Bacillota* (two from *Bacillus* and one from *Brevibacillus*), as well as *Bacteroidota* (formerly *Bacteroidetes*) (one from *Chryseobacterium*). In summary, most antagonistic bacterial strains from fungistatic and non-fungistatic soils belonged to the *Pseudomonadota* or *Bacillota* phyla, although with differences in species composition and their abundance, and their genomic features are presented in Table 10.

Table 10. Genomic features of the 23 antagonistic bacteria whose genomes have been sequenced in this study.

Species name from TYGS	Isolate name	Field of isolation	Genome size (bp)	Plasmid	GC-content (%)	No. contigs	Coding DNA sequences (CDS)
Isolates from fungistatic soils							
			***Bacillota* (formerly *Firmicutes*)**				
B. licheniformis	IT-74MI3	MI3	4,240,635	-	45.92	18	4619
Bacillus GS-1	IT-79MI2	MI2	5,465,265	+	35.44	139	6013
B. pseudomycoides	IT-19CA3	CA3	4,323,109	+	35.75	79	4603
B. pseudomycoides	IT-40CA3	CA3	3,061,249	+	35.83	36	3223
Priestia megaterium	IT-180MI3	MI3	5,635,521	+	37.87	44	6194
Priestia megaterium	IT-210MI2	MI2	5,379,042	+	37.85	28	5699
			***Pseudomonadota* (formerly *Proteobacteria*)**				
Kosakonia quasisacchari	IT-91MI3	MI3	5,073,466	-	53.48	70	4744
P. donghuensis	IT-53CA3	CA3	5,663,148	-	62.45	59	5408
P. chlororaphis	IT-51CA3	CA3	6,957,669	-	62.92	29	6655
P. chlororaphis	IT-162MI3	MI3	6,686,366	-	63.10	21	6340
Isolates from non-fungistatic soils							
			***Bacteroidota* (formerly *Bacteroidetes*)**				
Chryseobacterium GS-2	IT-36CA2	CA2	5,012,043	-	35.62	27	4631

***Bacillota* (formerly *Firmicutes*)**							
Brevibacillus GS-3	IT-7CA2	CA2	6,478,916	+	47.11	55	6303
B. licheniformis	IT-13CA1	CA1	4,332,481	-	45.75	54	4751
B. velezensis	IT-133MI5	MI5	3,857,335	-	46.57	38	3742
***Pseudomonadota* (formerly *Proteobacteria*)**							
Burkholderia GS-4	IT-111MI5	MI5	7,802,089	-	66.69	73	7775
Burkholderia ambifaria	IT-158MI4	MI4	7,617,524	-	66.61	75	7516
P. soli	IT-47CA2	CA2	5,708,236	-	63.78	72	5500
P. chlororaphis	IT-48CA2	CA2	6,818,347	-	62.98	60	6537
P. brassicacearum	IT-43CA1	CA1	6,737,027	-	60.86	70	6361
Pseudomonas GS-5	IT-194MI4	MI4	6,582,923	-	59.39	77	6261
Pseudomonas GS-6	IT-196MI5	MI5	6,303,596	-	59.61	84	6018
Pseudomonas GS-7	IT-93MI4	MI4	6,106,124	-	60.32	58	5645
Pseudomonas GS-8	IT-232MI5	MI5	6,512,142	-	59.15	61	6073

Furthermore, digital DNA-DNA hybridization values (computed with GGDC 3.0 and formula 2) of the 23 strains with their closest described type strains (available at the TYGS database; Meier-Kolthoff and Göker, 2019; Meier-Kolthoff et al., 2022) revealed eight novel genomospecies (hereafter termed *GS-1* to *GS-8*; Table 11) based on dDDH values below the 70% threshold for species delineation (Chun et al., 2018). Strains with dDDH values >70% were the following: *Bacillus licheniformis* IT-74MI3, *Bacillus pseudomycoides* IT-19CA3, *Bacillus pseudomycoides* IT-40CA3, *Priestia megaterium* IT-180MI3, *Priestia megaterium* IT-210MI2, *Kosakonia quasisacchari* IT-91MI3, *Pseudomonas donghuensis* IT-53CA3, *Pseudomonas chlororaphis* IT-51CA3, *Pseudomonas chlororaphis* IT-162MI3, *Bacillus licheniformis* IT-13CA1, *Bacillus velezensis* IT-133MI5, *Burkholderia ambifaria* IT-158MI4, *Pseudomonas soli* IT-47CA2, *Pseudomonas chlororaphis* IT-48CA2 and *Pseudomonas brassicacearum* IT-43CA1.

Table 11. Digital DNA-DNA hybridization (dDDH) values of the eight sequenced antagonistic strains and their closest described species (available in the TYGS database), whose dDDH values were below the 70%, a recommended cut-off value for bacterial species delineation. dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0; Meier-Kolthoff and Göker, 2019; Meier-Kolthoff et al., 2022), using the recommended BLAST method, and formula 2.

Species name from TYGS	**Field**	*B. pseudomycoides* DSM 12442	*Chryseobacterium aureum* 17S1E7	*Brevibacillus porteri* NRRL B-41110	*Burkholderia pyrrocinia* DSM 10685	*P. farris* SWRI79	*P. jessenii* DSM 17150	*P. koreensis* LMG 21318	*P. germanica* FIT 28
Isolates from fungistatic soils									
Bacillus GS-1 IT-79MI2	MI2	68.4							

Isolates from non-fungistatic soils										
Chryseobacterium GS-2 IT-36CA2	CA2	35.1								
Brevibacillus GS-3 IT-7CA2	CA2		58.7							
Burkholderia GS-4 IT-111MI5	MI5			61.7						
Pseudomonas GS-5 IT-194MI4	MI4				43.2					
Pseudomonas GS-6 IT-196MI5	MI5					48.3				
Pseudomonas GS-7 IT-93MI4	MI4						43.5			
Pseudomonas GS-8 IT-232MI5	MI5							48.0		

5.6.3. Presence of genes involved in biocontrol and plant growth promotion in antagonistic rhizosphere bacteria

After the genome sequencing, genomes of the 23 strains were annotated, searching for genes involved in biocontrol and plant-growth promotion.

The 13 genome-sequenced antagonistic *Pseudomonadota* included 10 *Pseudomonas* strains. Genes encoding the production of HCN, pyoverdine, extracellular alkaline protease, ethylene, auxin, the conversion of 2,3-butanediol to acetoin and further acetoin catabolism, and the phosphate solubilization and denitrification were evidenced in all 10 *Pseudomonas* strains, regardless of whether they originated from fungistatic or non-fungistatic soils (Table 12). *P. brassicacearum* IT-43CA1 (from non-fungistatic soil) carried genes encoding the production of DAPG (the presence of the whole operon was confirmed by antiSMASH) and ACC deaminase, whereas the three *P. chlororaphis* strains (from fungistatic or non-fungistatic soils) displayed genes which encode the production of phenazine, 2-hexyl-5-propyl-alkylresorcinol and pyrrolnitrin. In addition, *P. chlororaphis* IT-48CA2 (from non-fungistatic soil) had the gene encoding the insect-toxin FitD. In the two *Burkholderia* strains (both from a non-fungistatic soil), genes for synthesis of pyrrolnitrin, ACC deaminase, conversion of 2,3-butanediol to acetoin, acetoin catabolism and phosphate solubilization, were found.

Nine *Bacillota* strains were sequenced. Gene *alsSD* encoding acetoin biosynthesis was found in *Bacillus licheniformis* IT-74MI3 (from fungistatic soil), *Bacillus licheniformis* IT-13CA1 and *Bacillus velezensis* IT-133MI5 (both from non-fungistatic soil), whereas gene *ydjL* which encodes acetoin reductase/2,3-butanediol dehydrogenase was detected in *Bacillus velezensis* IT-133MI5 and *Brevibacillus GS-3* IT-7CA2 (both from non-fungistatic soils). The *Bacteroidota Chryseobacterium GS-2* IT- 36CA2 (from non-fungistatic soil) did not possess any of the genes investigated.

Table 12. Distribution of genes involved in biocontrol and plant-growth promotion in the 23 bacterial isolates studied. Presence of the property (the whole gene cluster) is marked with +, and when for certain property there are several possible pathways to achieve a function, names of the genes found in the genome are indicated. Genes were found with DIAMOND blastp (v.2.0.8.146; Buchfink et al., 2015), using the options --query-cover 80 --id 70 (query coverage >80%; amino acid identity >70%), if not specified otherwise.

Isolate name	Phenazine production	HPR production	2,4-DAPG production	Pyrrolnitrin production	HCN production	Pyoverdine production	Ethylene production	ACC deaminase	Auxin biosynthesis	Acetoin biosynthesis	2,3-butanediol biosynthesis	2,3-butanediol conversion to acetoin	Acetoin catabolism	Phosphate solubilization	Nitrogen fixation	Denitrification	Alkaline metalloproteinase production	Insect-toxin FitD
Isolates from fungistatic soils																		
Bacillus licheniformis																		
IT-74MI3										*alsSD*								
Bacillus GS-1																		
IT-79MI2																		
Bacillus pseudomycoides																		
IT-19CA3																		
IT-40CA3																		
Priestia megaterium (formerly *Bacillus megaterium*)																		
IT-180MI3																		
IT-210MI2																		
Kosakonia quasisacchari																		
IT-91MI3										*budBA*	*budBAC*				*+**			
Pseudomonas donghuensis																		
IT-53CA3					*+**				*ipdC*			*adh*	+	*gcd*		*nirS*		
Pseudomonas chlororaphis																		
IT-51CA3	+	+		+	+	+	+		*iaaMH*			*bdhA, adh*	+	*gcd, gad*		*nirK*	+	
IT-162MI3	+	+		+	+	+	+		*iaaMH*			*bdhA, adh*	+	*gcd, gad*		*nirK*	+	

Isolates from non-fungistatic soils																			
Chryseobacterium GS-2																			
IT-36CA2																			
Brevibacillus GS-3																			
IT-7CA2												ydjL							
Bacillus licheniformis																			
IT-13CA1									*alsSD*										
Bacillus velezensis																			
IT-133MI5									*alsSD*	*alsSD, ydjL*		*ydjL*							
Burkholderia GS-4																			
IT-111MI5				*+*			*+*					*adh*	*+**	*gad*					
Burkholderia ambifaria																			
IT-158MI4				*+*			*+*					*adh*	*+**	*gad*					
Pseudomonas soli																			
IT-47CA2					*+**			*ipdC*						*gad*		*nirK*			
Pseudomonas chlororaphis																			
IT-48CA2	*+*	*+*		*+*	*+*	*+*	*+*	*iaaMH*				*bdhA*		*gcd, gad*		*nirK*	*+*	*+*	
Pseudomonas brassicacearum																			
IT-43CA1			*+*		*+*			*+*	*iaaMH**			*adh*	*+*			*nirS*	*+*		
Pseudomonas																			
GS-5 IT-194MI4					*+*	*+*	*+*					*adh*	*+*	*gcd, gad*		*nirS*	*+*		
GS-6 IT-196MI5							*+*					*adh*		*gcd*					
GS-7 IT-93MI4					*+*	*+*	*+*							*gcd, gad*			*+*		
GS-8 IT-232MI5					*+*	*+*	*+*							*gcd, gad*			*+*		

Genes (and functions) that were searched for in the 23 bacterial isolates, but were not discovered: *pltABCDEFGLM* (production of pyoluteorin), *pchABCDEF* (production of pyochelin), *pmsABCE* (production of pseudomonine) and *iacABCDEFGHI* (auxin catabolism).

**hcnA* found with <70 % identity (63 % for isolate IT-47CA2 and 69 % for isolate IT-53CA3); *iaaH* found with only 33 % identity for isolate IT-43CA1; *acoX* and *acoR* found with 49 % and 57 % identity, respectively, for isolates IT-158MI4 and IT-111MI5; *nifD* and *nifK* found with 66 % and 49 % identity, respectively, in isolate IT-91MI3, but whole *nif* operon found in the genome.

Annotation of CAZymes showed that all the *Pseudomonas* genomes had genes encoding potential chitinases (except *Pseudomonas GS-6* IT-196MI5), as many as five in *P. chlororaphis* IT-51CA3 and IT-162M3 (from fungistatic soils) (Figure 20). *P. donghuensis* IT-53CA3, *P. chlororaphis* IT-51CA3 and IT-162MI3 (from fungistatic soils) and *P. soli* IT-47CA2, *P. chlororaphis* IT-48CA2, *Pseudomonas GS-6* IT-196MI5, *GS-7* IT-93MI4 and *GS-8* IT-232MI5 (from non-fungistatic soils) contained copies of the AA10 family, which includes lytic polysaccharide monooxygenases (LPMOs) that potentially target chitin (Figure 19). Genes coding for beta-glucanases were detected in four strains (from both fungistatic and non-fungistatic soils) and cellulase genes in *Pseudomonas GS-6* IT-196MI5 and *GS-5* IT-194MI4 (from non-fungistatic soils), but mannanase genes were not detected. The two *Burkholderia* strains (both from a non-fungistatic soil), displayed genes for potential chitinases (Figure 20), and *Burkholderia GS-4* IT-111MI5 exhibited genes for beta-glucanases and genes of the AA10 family (Figure 19). *Kosakonia quasisacchari* IT-91MI3 (from fungistatic soil) had a complete set of genes for synthesis of 2,3-butanediol, acetoin and nitrogenase, as well as genes encoding chitinases and especially cellulases (Figure 20). All the *Bacillota* strains presented genes encoding potential chitinases, up to six genes in *Brevibacillus GS-3* IT-7CA2 (from non-fungistatic soil) (Figure 19). *B. licheniformis* IT-74MI3 (from fungistatic soil), as well as *Brevibacillus GS-3* IT-7CA2, *B. licheniformis* IT-13CA1 and *B. velezensis* IT-133MI5 (from non-fungistatic soils), contained genes from the AA10 family (Figure 19). Genes coding for beta-glucanases were detected only in *B. velezensis* IT-133MI5 (from non-fungistatic soil), while cellulase genes were found in four strains, especially in *B. licheniformis* IT-74MI3 (from fungistatic soil) and *B. licheniformis* IT-13CA1 (from non-fungistatic soil). Mannanase genes were detected in *B. licheniformis* IT-74MI3 (from fungistatic soil), and in *B. licheniformis* IT-13CA1 and *B. velezensis* IT-133MI5 (from non-fungistatic soils). The *Bacteroidota Chryseobacterium GS-2* IT-36CA2 (from non-fungistatic soil) harbored genes encoding potential chitinases (Figure 20), genes belonging to the AA10 family and genes coding for potential beta-glucanases, but it did not possess any genes involved in cellulase or mannanase production (Figure 19).

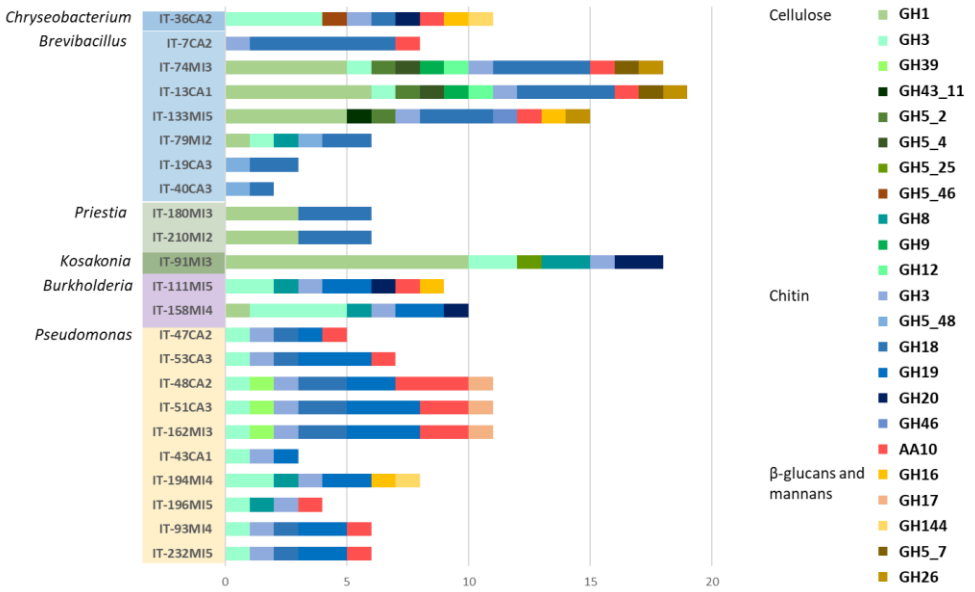

Figure 19. Abundance of genes belonging to CAZyme families potentially targeting cell wall components in fungi and oomycetes (cellulose, chitin, β-glucans and mannans), found in the genomes of sequenced bacterial isolates.

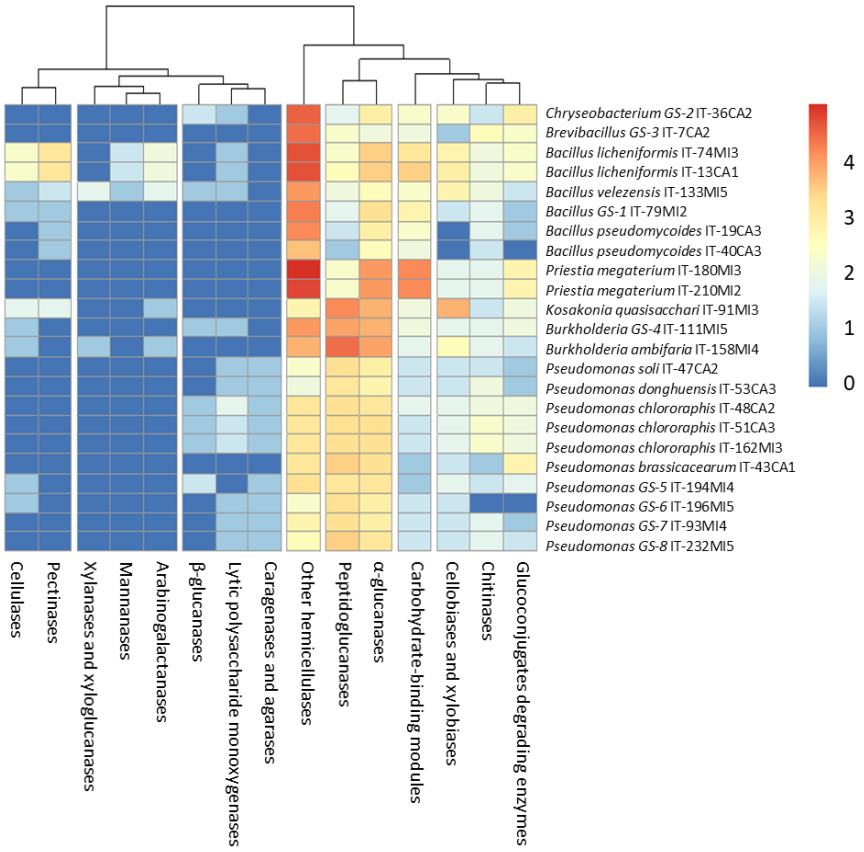

Figure 20. Heatmap showing the abundance of CAZyme genes annotated for each function found in the genomes of the 23 bacteria. Legend shows transformed counts.

In conclusion, isolates from both fungistatic and non-fungistatic soils possessed genes involved in biocontrol or plant-growth promotion. Distribution of phytobeneficial traits was, to a large extent, taxa-specific.

5.6.4. *In vitro* biocontrol and plant growth promoting activity of antagonistic rhizosphere bacteria and correspondence between gene presence and *in vitro* activities

After the genome annotation, functional characterization of biocontrol and plant-growth promoting activities of all of the sequenced strains was performed. This included the assessment of *in vitro* siderophore production, production of HCN, production of lytic enzymes (proteases, chitinases and cellulases), phytohormones production, ACC deaminase production and solubilization of phosphates (Figure 21).

In vitro production of HCN and siderophores was recorded in strains from both fungistatic and non-fungistatic soils, while solubilization of phosphates was recorded only in *Pseudomonas* and *Burkholderia* strains from non-fungistatic soils (Table 13). ACC deaminase activity was found in *Burkholderia* strains IT-111MI5 and IT-158MI4, and *P. brassicacearum* IT-43CA1 (all from non-fungistatic soils). Protease activity was detected in the majority of the strains tested, while cellulase activity was observed in only two fungistatic-soil strains (*B. licheniformis* IT-74MI3 and *P. megaterium* IT-210MI2) and one non-fungistatic-soil strain (*B. velezensis* IT-133MI5). Chitinase activity and production of IAA were, similarly to the protease activity, detected in almost all strains. Production of indole-3-pyruvic acid was found in *B. pseudomycoides* IT-40CA3 and that of

tryptophol in *P. megaterium* IT-180MI3 and *K. quasisacchari* IT-91MI3 (all three from fungistatic soils), and none of the strains produced indole-3-butyric acid, trans-zeatin riboside, kinetin, 6-benzylaminopurine, gibberellin A1, gibberellic acid or abscisic acid. All the remaining phytohormones tested, i.e., indole-3-lactic acid, indole-3-carboxylic acid, indole-3-propionic acid, trans-zeatin, isopentenyl adenosine and kynurenic acid, were produced by strains from both fungistatic and non-fungistatic soils.

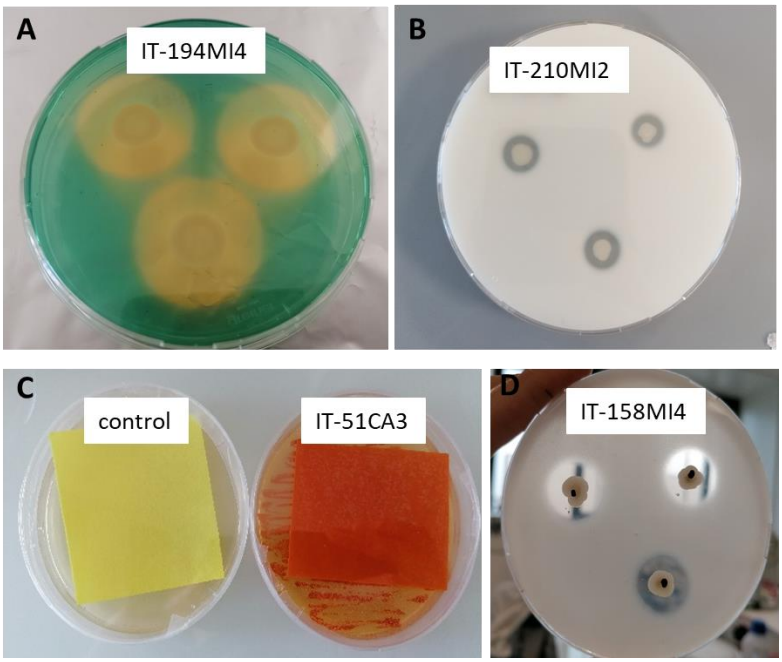

Figure 21. *In vitro* characterization of rhizosphere biocontrol strains. (A) *Pseudomonas GS-5* IT-194MI4 producing siderophores. (B) *Priestia megaterium* IT-210MI2 producing proteases. (C) Right: *Pseudomonas chlororaphis* IT-51CA3 producing HCN; Left: Negative control. (D) *Burkholderia ambifaria* IT-158MI4 with phosphates solubilizing activity.

Production of HCN was confirmed *in vitro* in eight of nine *Pseudomonas* strains (from both fungistatic and non-fungistatic soils) carrying *hcnABC* genes (Table 13). Siderophore production was evidenced in all *Pseudomonas* strains, six of which (from both types of soils) carrying the *pvdL* pyoverdine gene, but the two *Bacillus* strains (from fungistatic or non-fungistatic soil), the two *Burkholderia* isolates and *Chryseobacterium* sp. IT-36CA2 (all three from non-fungistatic soils) produced siderophores despite lacking the *pvdL* gene. Phosphate solubilization was confirmed in strains originating only from non-fungistatic soils, i.e., six *Pseudomonas* strains and two *Burkholderia* strains, but only some of them had glucose dehydrogenase gene *gad* and/or gluconate dehydrogenase gene *gcd*, while four *Pseudomonas* strains (three from fungistatic soils and one from non-fungistatic soil) had the genes but did not solubilize phosphate under the conditions tested. ACC deaminase activity was found in all *Burkholderia* strains and *P. brassicacearum* IT-43CA1 (all from non-fungistatic soils and carrying *acdS* gene). Protease activity was detected in almost all strains (from both types of soils), including the seven *Pseudomonas* strains carrying the *aprA* gene. Cellulase activity was observed in only two fungistatic-soil strains (*B. licheniformis* IT-74MI3 and *P. megaterium* IT-210MI2) and one non-fungistatic-soil strain (*B. velezensis* IT-133MI5). Chitinase activity was confirmed *in vitro* for 20 of 23 strains with genes encoding chitinases (and/or genes of the AA10 family, as in *Pseudomonas*

GS-6 IT-196MI5) and originating from fungistatic and non-fungistatic soils. As many as 18 strains (from both types of soils) produced IAA, even though the *iaaMH* or *ipdC* genes were found in only six *Pseudomonas* strains.

In summary, phosphate solubilization and ACC deaminase were recorded in strains from non-fungistatic soils, while production of indole-3-pyruvic acid and tryptophol was recorded only in strains originating from fungistatic soils, while the remaining tested traits were demonstrated in strains from both fungistatic and non-fungistatic soils.

Table 13. Correspondence between gene presence and *in vitro* activities involved in plant-growth promotion and biocontrol in 23 isolates, according to the soil fungistasis status. Activity is marked with a green colour. Gene corresponding to a given activity *in vitro* (when found in the genomes) is indicated. Cellulases and chitinases were predicted using dbCAN2 (v.3; Zhang et al., 2018) and compared with the CAZy database using HMMER (v.3.3; Eddy, 2011). Prediction of function and substrate specificity of CAZyme families or subfamilies was performed based on a review of activities assigned to CAZymes with known structures (characterized enzymes) in the CAZy database (http://www.cazy.org) (Lombard et al., 2014) and manually curated, as previously described (López-Mondéjar et al., 2022).

Bacterial isolate	HCN	Siderophores	Phosphate solubilization	ACC deaminase	Protease	Cellulase	Chitinase	Indole-3-acetic acid	Indole-3-lactic acid	Indole-3-carboxylic acid	Indole-3-pyruvic acid	Indole-3-butyric acid	Tryptophol	Indole-3-propionic acid	Trans-zeatin	Trans-zeatin riboside	Kinetin	6-Benzylaminopurine	Isopentenyl adenosine	Gibberellin A1	Gibberellic acid	Abscisic acid	Kynurenic acid
Isolates from fungistatic soils																							
Bacillus licheniformis																							
IT-74MI3						+	+																
Bacillus																							
GS-1 IT-79MI2						+	+																
Bacillus pseudomycoides																							
IT-19CA3							+																
IT-40CA3							+																
Priestia megaterium (formerly *Bacillus megaterium*)																							
IT-180MI3							+																
IT-210MI2							+																
Kosakonia quasisacchari																							
IT-91MI3						+	+																
Pseudomonas donghuensis																							
IT-53CA3	+*		*gcd*				+	*ipdC*															
Pseudomonas chlororaphis																							
IT-51CA3	+	+	*gcd, gad*		+		+	*iaaMH*															

IT-162MI3	+	+	*gcd, gad*		+		+	*iaaMH*
Isolates from non-fungistatic soils								
Chryseobacterium								
GS-2 IT-36CA2							+	
Brevibacillus								
GS-3 IT-7CA2							+	
Bacillus licheniformis								
IT-13CA1						+	+	
Bacillus velezensis								
IT-133MI5						+	+	
Burkholderia								
GS-4 IT-111MI5			*gad*	+		+	+	
Burkholderia ambifaria								
IT-158MI4			*gad*	+		+	+	
Pseudomonas soli								
IT-47CA2	+*		*gad*				+	*ipdC*
Pseudomonas chlororaphis								
IT-48CA2	+	+	*gcd, gad*		+		+	*iaaMH*
Pseudomonas brassicacearum								
IT-43CA1	+			+	+		+	*iaaMH* *
Pseudomonas								
GS-5 IT-194MI4	+	+	*gcd, gad*		+	+	+	
GS-6 IT-196MI5			*gcd*			+		
GS-7 IT-93MI4	+	+	*gcd, gad*		+		+	
GS-8 IT-232MI5	+	+	*gcd, gad*		+		+	

**hcnA* found with <70 % identity (63 % for isolate IT-47CA2 and 69 % for isolate IT-53CA3); *iaaH* found with only 33 % identity in isolate IT-43CA1

5.6.5. Inhibitory effect of bacterial volatile organic compounds (VOCs) on growth and inhibitory effect of bacterial exudates on sporulation of *Fusarium graminearum* Fg1

In this part of the study, the inhibitory effect of VOCs produced by rhizosphere isolates towards *F. graminearum* Fg1, as well as the ability of bacterial isolates to inhibit sporulation of *F. graminearum* Fg1 in liquid medium, were tested. Ability of bacterial isolates to inhibit mycelial growth of *F. graminearum* Fg1 by VOCs was only seen in four antagonistic strains, i.e., *P. soli* IT-47CA2 (by 47.1%) (Figure 22), *Pseudomonas GS-5* IT-194MI4 (by 23.5%), and *Burkholderia ambifaria* IT-158MI4 (by 41.2%) and *Burkholderia GS-4* IT-111MI5 (by 11.8%). All four originated from non-fungistatic soils.

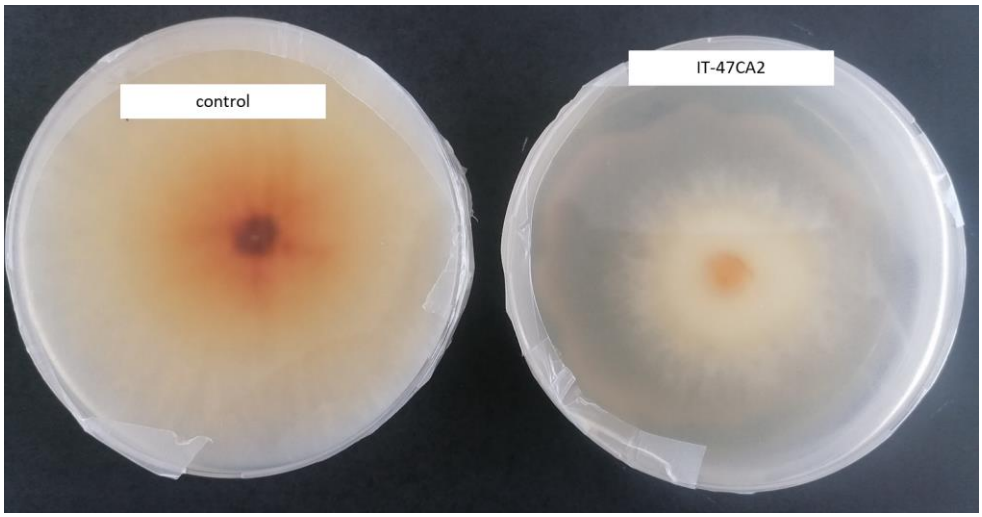

Figure 22. Ability of *Pseudomonas soli* IT-47CA2 to inhibit mycelial growth of *Fusarium graminearum* Fg1 through production of VOCs. Right: *Pseudomonas soli* IT-47CA2 inhibiting growth of *Fusarium graminearum* Fg1; Left: Negative control, i.e., *Fusarium graminearum* Fg1 only.

Additionally, a microplate assay was used to test the ability of bacterial exudates to inhibit conidia germination of *F. graminearum* Fg1. Conidia germination was inhibited by exudates of *P. donghuensis* IT-53CA3 (from fungistatic soil) by 75 %, and *Burkholderia GS-4* IT-111MI5 (from non-fungistatic soil) by 26.6 %. The following nine strains (from fungistatic or non-fungistatic soils) also inhibited conidia germination, but at levels <20%, i.e., *Chryseobacterium GS-2* IT-36CA2 (17.2%), *Brevibacillus GS-3* IT-7CA2 (13.7%), *B. licheniformis* IT-74MI3 (11.1%), *B. licheniformis* IT-13CA1 (11.2%), *Bacillus GS-1* IT-79MI2 (16.3%), *B. pseudomycoides* IT-19CA3 (14.4%) and IT-40CA3 (11.3%), *P. megaterium* IT-210MI2 (10.8%) and *B. ambifaria* IT-158MI4 (18.7%).

In summary, VOCs of certain antagonistic strains (from non-fungistatic soils only) affected mycelial growth of *F. graminearum* Fg1. In contrast, exudates of several strains from fungistatic and non-fungistatic soils inhibited conidia germination.

5.6.6. Additional genomic analyses of the most promising antagonistic rhizosphere bacteria

Besides genomic and *in vitro* characterization of rhizospheric strains, total number of putative biosynthetic gene clusters (BGCs) in each rhizospheric strain was also identified, using the antiSMASH (Blin et al., 2019) within the MicroScope platform. Therefore, up to 20 BGCs in *B. ambifaria* IT-158MI4 and 19 in *B. velezensis* IT-133MI5 (both from non-fungistatic soils) were

identified (Table 14). The highest number of completed BGCs was 11 (in *B. velezensis* IT-133MI5 from non-fungistatic soil). The highest number of BGCs in isolates from fungistatic soils was respectively 16 and 15 for *P. chlororaphis* IT-51CA3 and IT-162MI3, both with three completed BGCs.

Table 14. Number of putative biosynthetic gene clusters (BGCs) and number of BGCs with completion 1 or 1*, in the 23 studied bacterial isolates, found using the antiSMASH (Blin et al., 2019) within the MicroScope platform.

Species name from TYGS	Bacterial isolate	Number of putative BGCs	Number of BGCs with completion 1	Number of BGCs with completion 1*
Isolates from fungistatic soils				
Bacillus licheniformis	IT-74MI3	12	3	0
Bacillus GS-1	IT-79MI2	13	2	0
Bacillus pseudomycoides	IT-19CA3	5	1	0
Bacillus pseudomycoides	IT-40CA3	2	1	0
Priestia megaterium	IT-180MI3	6	0	0
Priestia megaterium	IT-210MI2	7	0	0
Kosakonia quasisacchari	IT-91MI3	6	1	0
Pseudomonas donghuensis	IT-53CA3	5	0	0
Pseudomonas chlororaphis	IT-51CA3	16	1	2
Pseudomonas chlororaphis	IT-162MI3	15	1	2
Isolates from non-fungistatic soils				
Chryseobacterium GS-2	IT-36CA2	9	0	0
Brevibacillus GS-3	IT-7CA2	15	1	0
Bacillus licheniformis	IT-13CA1	12	3	0
Bacillus velezensis	IT-133MI5	19	11	0
Burkholderia GS-4	IT-111MI5	16	3	0
Burkholderia ambifaria	IT-158MI4	20	3	0
Pseudomonas soli	IT-47CA2	14	3	3
Pseudomonas chlororaphis	IT-48CA2	16	2	2
Pseudomonas brassicacearum	IT-43CA1	11	2	0
Pseudomonas GS-5	IT-194MI4	12	0	0
Pseudomonas GS-6	IT-196MI5	9	0	0
Pseudomonas GS-7	IT-93MI4	10	0	1
Pseudomonas GS-8	IT-232MI5	11	0	1

* When two or more genes in a single MIBiG (The Minimum Information about a Biosynthetic Gene cluster database) curated region were similar, the same gene in MicroScope database can hit on these MIBiG genes. When this happens, the completion can be higher than 1 (represented by 1*).

Subsequently, the most promising antagonistic bacteria, i.e., those that inhibited *F. graminearum* Fg1 mycelial growth in a dual-culture assay, those that altered fungal colony morphology, and those that produced VOCs inhibiting fungal mycelial growth and/or whose exudates inhibited conidia germination (Figure 23) were chosen for further analysis. They included *Brevibacillus GS-3* IT-7CA2, *B. velezensis* IT-133MI5, *B. ambifaria* IT-158MI4, *P. soli* IT-47CA2, *P. chlororaphis* IT-48CA2 and *Pseudomonas GS-5* IT-194MI4 from non-fungistatic-soils, but only *P. donghuensis* IT-53CA3 from fungistatic soil. On one hand, BGCs found in their genomes were analyzed and manually curated. On the other hand, in *planta* assay in the presence of *F. graminearum* Fg1 and bacterial strains was performed.

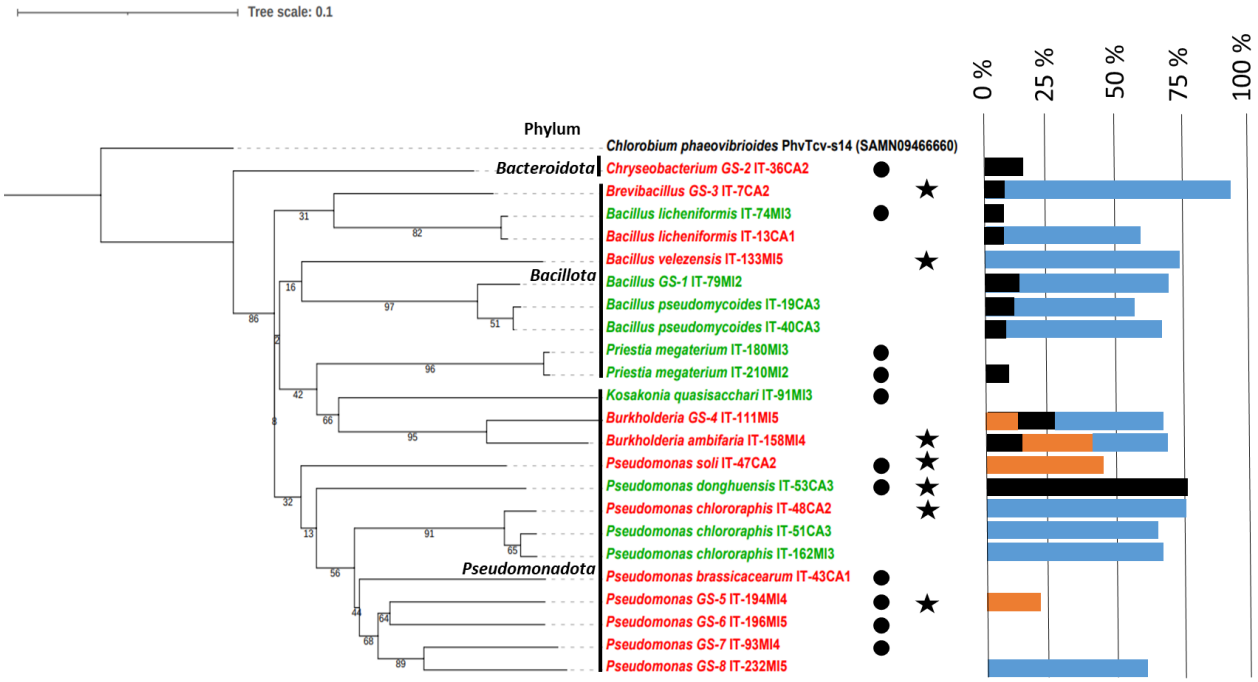

Figure 23. Whole genome-based phylogenetic tree for 23 antagonistic bacteria from fungistatic (green) and non-fungistatic soils (red), and their ability to affect *Fusarium graminearum* Fg1 colony morphology (black circle), inhibit *Fusarium graminearum* Fg1 conidia germination (black bars), inhibit *Fusarium graminearum* Fg1 via VOCs (orange bars), and inhibit *Fusarium graminearum* Fg1 mycelial growth in a dual-culture assay (blue bars). Black stars indicate isolates chosen for *in planta* assay and those whose BGCs found in the genome were manually curated. The tree was inferred with FastME 2.1.6.1 (Lefort et al., 2015) from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences, and visualized using iTOL software (Letunic and Bork, 2021). Branch numbers are GBDP pseudo-bootstrap support values from 100 replications, with an average branch support of 56.9 %. *Chlorobium phaeovibrioides* PhvTcv-s14 (BioSample accession number: SAMN09466660) was used as the outgroup.

Manual curation of BGCs found in the seven genomes of chosen biocontrol bacterial strains showed that *Brevibacillus* sp. *GS-3* IT-7CA2 harbored gene clusters coding for antibiotics, such as edeine, tyrocidin (surfactin), lipopeptide antibiotic, linear gramicidin, bacillaene-like antifungal product, and siderophores (Table 15). In the genome of *B. velezensis* IT-133MI5, BGCs were found involved in the production of antibiotics (mycosubtilin, macrolactin, plipastatin, difficidin, mersacidin, surfactin, bacilysin, lanthipeptides and bacillibactin), while *B. ambifaria* IT-158MI4 had the potential of producing phenazine-like compound, pyrrolnitrin (as confirmed by BLAST), non-ribosomal antifungal oligopeptides, as well as the siderophores enterobactin and ornibactin. *P. chlororaphis* IT-48CA2 possessed genes for phenazine, pyrrolnitrin (as confirmed by BLAST), putative bacitracin/enterobactin, mangotoxin and different siderophores. *P. donghuensis* IT-53CA3 had BGCs for pyoverdine and mangotoxin biosynthesis, while *P. soli* IT-47CA2 had BGCs for production of cyclic lipopeptide xantholysin, dapdiamides, mangotoxin and siderophores. Finally, *Pseudomonas GS-5* IT-194MI4 had the potential of producing mangotoxin and different siderophores. In summary, the genomes of these seven strains displayed BGCs putatively coding for the production of siderophores and antibiotics, potentially involved in biocontrol.

Table 15. List of secondary metabolites identified using the antiSMASH (Blin et al., 2019) and manually curated in *Brevibacillus GS-3* IT-7CA2, *Bacillus velezensis* IT-133MI5, *Burkholderia ambifaria* IT-158MI4, *Pseudomonas chlororaphis* IT-48CA2, *Pseudomonas donghuensis* IT-53CA3, *Pseudomonas soli* IT-47CA2 and *Pseudomonas GS-5* IT-194MI4. The start, end, length and region type of the predicted biosynthetic gene cluster are shown. Abbreviations: transAT-PKS (trans-acyltransferase polyketide synthases), NRPS (non-ribosomal peptide synthetases), T3PKS (type III polyketide synthases), LAP (linear azol(in)e-containing peptides), T1PKS (type I polyketide synthases), hserlactone (homoserine lactone), PpyS-KS (PPY-like pyrone) and NAGGN (N-acetylglutaminylglutamine amides).

Isolate	Start	End	Length	Region type	Pathway manually curated
Brevibacillus GS-3 IT-7CA2	49394	126050	76657	transAT-PKS, NRPS	Edeine
	145711	204801	59091	NRPS, transAT-PKS-like	Subtilisin-like alkaline serine protease
	453835	494899	41065	T3PKS	Spore germination factor
	602938	624920	21983	terpene	Lipopolysacharide synthesis, terpene synthesis, sporulation related
	759927	867046	107120	NRPS, transAT-PKS-like, transAT-PKS	Bacillaene like, natural product with antifungal properties
	1389577	1413147	23571	LAP, bacteriocin	Maturation of compound from a ribosomally produced precursor polypeptide
	1522244	1591382	69139	NRPS	Tyrocidin (surfactin) synthesis
	2279275	2346852	67578	NRPS	Lipopeptide antibiotic synthesis
	2926082	2936930	10849	bacteriocin	Encapsulins
	3197643	3258150	60508	NRPS	Siderophore or antibiotic
	4892513	4915134	22622	lanthipeptide	Lanthipeptide involved in spore germination
	4989239	5042547	53309	NRPS	Anabaenopeptin NZ 857 / nostamide A
	5119171	5187480	68310	lanthipeptide, NRPS	Linear gramicidin synthetase
	5361405	5375121	13717	siderophore	Petrobactin siderophore
	6316909	6319581	2673	NRPS	Unknown
B. velezensis IT-133MI5	1	79202	79202	betalactone, NRPS, transAT-PKS	Mycosubtilin
	143184	252423	109240	transAT-PKS-like, transAT-PKS, NRPS, T3PKS	Bacillaene/alkaline serine protease aprX
	474237	562458	88222	transAT-PKS	Macrolactin
	865867	886607	20741	terpene	Unknown
	969936	1011180	41245	PKS-like	Polyketide
	1075641	1113576	37936	NRPS	
	3824989	3834225	9237	NRPS	Plipastatin
	3834326	3842245	7920	NRPS	
	1138866	1160749	21884	terpene	Sesquarterpenes
	1222008	1263108	41101	T3PKS	Antibiotic
	1378699	1484878	106180	transAT-PKS-like, transAT-PKS	Difficidin
	2319521	2342709	23189	lanthipeptide	Mersacidin
	2694377	2719769	25393	NRPS	
	3815603	3824888	9286	NRPS	Surfactin
	2941791	2969557	27767	NRPS	
	2738042	2779460	41419	other	Bacilysin synthesis

	3068358	3090973	22616	lanthipeptide	Lanthipeptide
	3336546	3374388	37843	NRPS	Bacillibactin
	3484796	3495134	10339	bacteriocin	Circular bacteriocins, antimicrobial peptides
B. ambifaria IT-158MI4	246251	293810	47560	T1PKS	Putative heparinase II/III family protein
	782932	827843	44912	T1PKS, NRPS-like	Unknown
	1195618	1216259	20642	hserlactone	Acyl-homoserine-lactone synthase, involved in quorum sensing
	1361820	1408642	46823	NRPS	Enterobactin like siderophore
	1623867	1644856	20990	terpene	Unknown
	2304168	2345853	41686	phosphonate	Unknown
	2380170	2425048	44879	arylpolyene	Unknown
	2480912	2501976	21065	terpene	Non-heme iron decarboxylase, involved in antibiotic novobiocin synthesis
	2707158	2748381	41224	arylpolyene	Cardiolipin synthase C
	3554068	3574496	20429	phenazine	Phenazine like compound
	3934141	3944956	10816	bacteriocin	Nanocompartment encapsulin Linocin M18
	4441363	4485247	43885	T1PKS	Adhesin bpaC, virulence factor, biofilm formation
	4643983	4654369	10387	ectoine	Partial ectoine synthesis pathway
	6250299	6260697	10399	ectoine	Partial ectoine synthesis pathway
	4708504	4732599	24096	terpene	Squalene biosynthesis
	4918184	4972895	54712	NRPS	Siderophore ornibactin synthesis
	5778732	5819817	41086	other	Pyrrolnitrin
	5941856	5963904	22049	terpene	Unknown
	6000086	6020691	20606	hserlactone	Unknown lipopeptides
	6555814	6641248	85435	NRPS, T1PKS	Non-ribosomal oligopeptides with antifungal activity
P. chlororaphis IT-48CA2	409268	428234	18967	siderophore	Polycarboxylate siderophore staphyloferrin B, IucA/IucC
	648898	675789	26892	terpene	Beta-caryophyllene-like sesquiterpenoid
	967567	1011187	43621	arylpolyene	Lipoprotein
	1778015	1809062	31048	NRPS	Pyoverdine synthesis
	1997392	2020639	23248	betalactone	Biotin synthesis pathway, fatty acid (long saturated)
	3632874	3643770	10897	bacteriocin	Unknown
	4180078	4190971	10894	bacteriocin	Unknown lipoprotein
	4613843	4636461	22619	phenazine, hserlactone	Phenazine
	4691104	4711784	20681	hserlactone	Quorum sensing involved pathway 2 *psy*
	5140419	5161078	20660	hserlactone	Quorum sensing involved pathway 1 *rhl*
	5313688	5372696	59009	NRPS	Putative bacitracin/enterobactin
	5885184	5938200	53017	NRPS	Pyoverdine synthetase
	6073140	6119367	46228	NRPS, resorcinol	Pyoverdine synthetase
	6689686	6720468	30783	NRPS-like	Mangotoxin biosynthesis
	6720569	6749771	29203	other	Pyrrolnitrin
	6790107	6793135	3029	NRPS	Massetolide, orfamide, syringopeptin like synthesis
P. donghuensis IT-53CA3	1138554	1206048	67495	NRPS	Pyoverdine
	3059651	3112613	52963	NRPS	Pyoverdine
	3550188	3593025	42838	NRPS-like	Secondary metabolite
	5009796	5053409	43614	arylpolyene	Lipoprotein
	5583820	5605556	21737	NRPS-like	Mangotoxin biosynthesis
P. soli IT-47CA2	1814889	1842944	28056	NRPS	
	1843049	1880909	37861	NRPS	Xantholysin (cyclic lipopeptides)
	2525370	2598975	73606	NRPS	
	2012633	2078210	65578	NRPS	Pseudomonine heterocyclic siderophore

	2201021	2242208	41188	T3PKS	Mevalonate pathway isoprenoide synthesis
	2278901	2331848	52948	NRPS	Pyoverdine synthetase A
	2495147	2516196	21050	PpyS-KS	Pseudopyronines A and B
	2701819	2712640	10822	bacteriocin	Unknown
	4017294	4049445	32152	NRPS	Lipopeptide siderophores
	5549990	5559838	9849	NAGGN	Dapdiamides, tripeptide antibiotics
	5611199	5626725	15527	NRPS-like	Mangotoxin biosynthesis
	5654779	5666507	11729	NRPS	
	5666608	5672935	6328	NRPS	Siderophore
	5681332	5684101	2770	NRPS	
Pseudomonas GS-5 IT-194MI4	23130	36540	13411	butyrolactone	Unknown
	517198	528034	10837	bacteriocin	Cellular processes, signalling transduction
	2172527	2181555	9029	bacteriocin	Unknown
	2209373	2221343	11971	bacteriocin	Unknown
	2376537	2388459	11923	siderophore	Unknown
	2984871	3061145	76275	NRPS	Pyoverdine synthesis
	3186077	3213173	27097	betalactone	Biotin like synthesis pathway, fatty acid (long saturated)
	3511162	3564145	52984	NRPS	Pyoverdine synthetase A
	3719392	3734144	14753	NAGGN	N-acetylglutaminylglutamine synthetase/cell wall synthesis
	3932490	3976095	43606	arylpolyene	Lipoprotein
	5315442	5326287	10846	bacteriocin	Cardiolipin synthase A
	6372519	6389175	16657	NRPS-like	Mangotoxin biosynthesis

5.6.7. *In planta* effects of selected antagonistic bacteria on wheat inoculated with *Fusarium graminearum* Fg1

The same antagonistic bacterial strains whose BGCs found in genomes were manually curated, were also chosen for *in planta* phytoprotection assay. They included: *Brevibacillus GS-3* IT-7CA2, *B. velezensis* IT-133MI5, *B. ambifaria* IT-158MI4, *P. soli* IT-47CA2, *P. chlororaphis* IT-48CA2, *Pseudomonas GS-5* IT-194MI4 and *P. donghuensis* IT-53CA3 (Figure 24). In the plant assay performed with the soil LCSA, the addition of *F. graminearum* Fg1 alone resulted in a significantly lower number of germinated seeds at 14 days (Figure 24A), high disease symptoms (Figure 24B), lower biomass (Figure 24C) and lower chlorophyll rate at 45 days (Figure 24D), in comparison with non-inoculated seeds. In comparison with seeds inoculated with *F. graminearum* Fg1, there was a trend of a higher number of germinated seeds when inoculation was carried out with three of seven bacteria, i.e., *B. ambifaria* IT-158MI4 and *Pseudomonas GS-5* IT-194MI4 (trend significant at $P < 0.05$) from non-fungistatic soils, and *P. donghuensis* IT-53CA3 from fungistatic soil. In addition, bacterial inoculation resulted in lower disease symptoms with *B. velezensis* IT-133MI5, *P. soli* IT-47CA2, *P. chlororaphis* IT-48CA2, *Pseudomonas* sp. *GS-5* IT-194MI4 and *P. donghuensis* IT-53CA3. Finally, biomass was lower with *B. ambifaria* IT-158MI4 and the four *Pseudomonas* strains, and chlorophyll rate of germinated plants was lower with *B. ambifaria* IT-158MI4 and *Pseudomonas* sp. *GS-5* IT-194MI4.

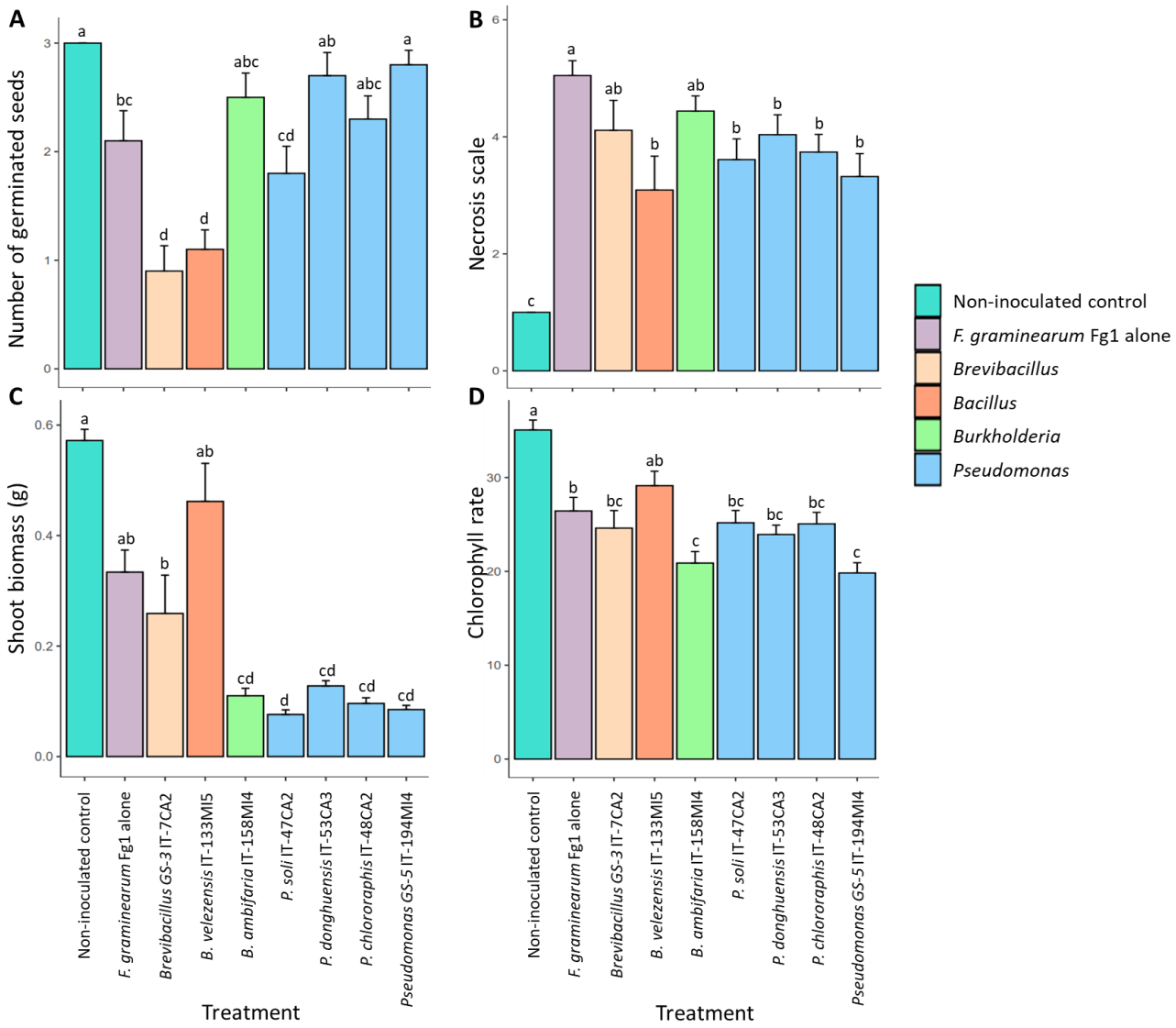

Figure 24. Results of the *in planta* protection assay. (A) Number of germinated seeds at two weeks after inoculation with antagonistic bacteria and *Fusarium graminearum* Fg1. Results are presented as mean + standard error. Data were analyzed using Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with letters a to d. (B) Disease symptoms of crown-rot at 45 days after inoculation with antagonistic bacteria and *Fusarium graminearum* Fg1. Non-germinated plants were regarded as missing data (NA). Results are presented as mean + standard error. Data were analyzed using Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with significance letters a to c. (C) Shoot biomass of wheat plants at 45 days after inoculation with antagonistic bacteria and *Fusarium graminearum* Fg1. Non-germinated plants were regarded as missing data (NA). Results are presented as mean + standard error. Data were analyzed using Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with letters a to d. (D) Chlorophyll rate of wheat plants at 45 days after inoculation with antagonistic bacteria and *Fusarium graminearum* Fg1. The chlorophyll rate of each wheat plant was the average of three measurements, taken on the 5th, 6th and 7th grown leaf. Non-germinated plants and plants without grown leaves were regarded as missing data (NA). Results are presented as mean + standard error. Data were analyzed using ANOVA and Tukey's test ($P < 0.05$), and statistical differences are shown with letters a to c.

In summary, *Pseudomonas GS-5* IT-194MI4 enhanced wheat germination and conferred protection from crown-rot disease, but at the expense of shoot biomass and chlorophyll rate. The three other *Pseudomonas* strains and *B. velezensis* IT-133MI5 conferred some protection, but without improving seed germination, while the three *Pseudomonas* strains also affected the shoot biomass.

5.7. Indigenous *Pseudomonas* in soils suppressive to *Fusarium graminearum*

Besides isolating bacteria of contrasting taxonomy with biocontrol activity against *F. graminearum* Fg1 from fungistatic vs. non-fungistatic soils, the third objective of this research was to identify the genomic and functional particularities of *Pseudomonas* bacteria isolated from suppressive vs. non-suppressive soils. This was motivated by the fact that *Pseudomonas* has an important role in suppressive soils, harbors genes potentially involved in biocontrol, as shown in section 5.6.3., nevertheless comparison of *Pseudomonas* from suppressive and non-suppressive soils has not been extensively studied so far, although it may provide insight into the functioning of suppressive soils. For this phase of research, rhizospheres of wheat plants grown in soils from Mionica (MI4 (non-suppressive), MI5, MI2 and MI3 (suppressive)) were used, inoculated or not inoculated with *F. graminearum* Fg1, harvested after the supressiveness assay, previously described in section 5.3.

5.7.1. Microbiota diversity in soils from Mionica analyzed through *rpoD* metabarcoding

Since *Pseudomonas* subcommunities may be different in suppressive vs. non-suppressive soils, in this part of the study, *rpoD* metabarcoding of four soils from Mionica was performed. In these datasets, the rarefaction curves tended to reach a plateau, indicating that the sequencing method supplied sufficient sequences to cover most of the diversity (Supplementary material; Chapter 4B; Figure S2). Metabarcoding data are deposited into the EBI/EMBL database under the accession number PRJEB61447. The differences in Chao1 index (a measure of species richness) between the four soils were not significant, however, there were differences in the inverse Simpson (a measure of species diversity and evenness), Shannon (a measure of species diversity) and Observed (a measure of species richness) indices between the MI2 and MI3 soils, that were both fungistatic and suppressive ($P<0.05$) (Figure 25). Inverse Simpson index was significantly different in soils MI5 and MI3, compared to soils MI4 and MI2, and the situation was similar with Shannon index, while the Observed index was significantly different in soils MI4, MI5 and MI3, compared to soil MI2. Altogether, species diversity was higher in soils MI5 and MI3 than in soils MI4 and MI2 (as measured with inverse Simpson and Shannon indices), while species richness was similar in all four soils, when measured with Chao1 index, but it was lower in soil MI2 when measured with Observed index, which does not take into the account the rare species.

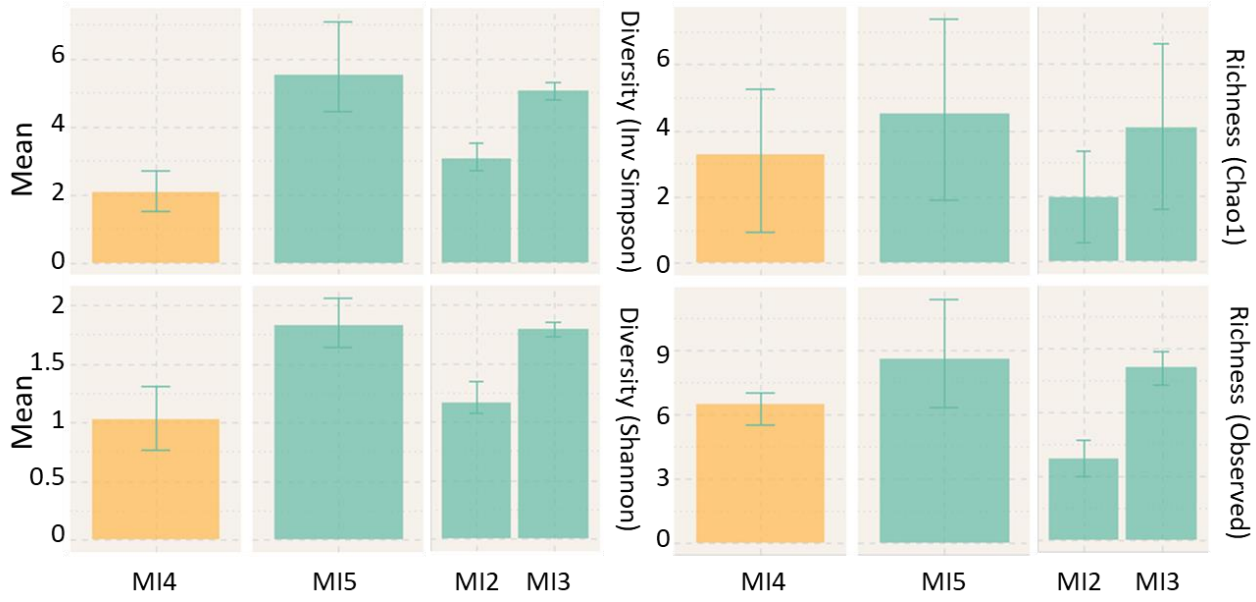

Figure 25. α-Diversity (boxplots) of *Pseudomonas* communities in soils MI4, MI5, MI2 and MI3. Vertical bars represent 95% confidence intervals of the mean.

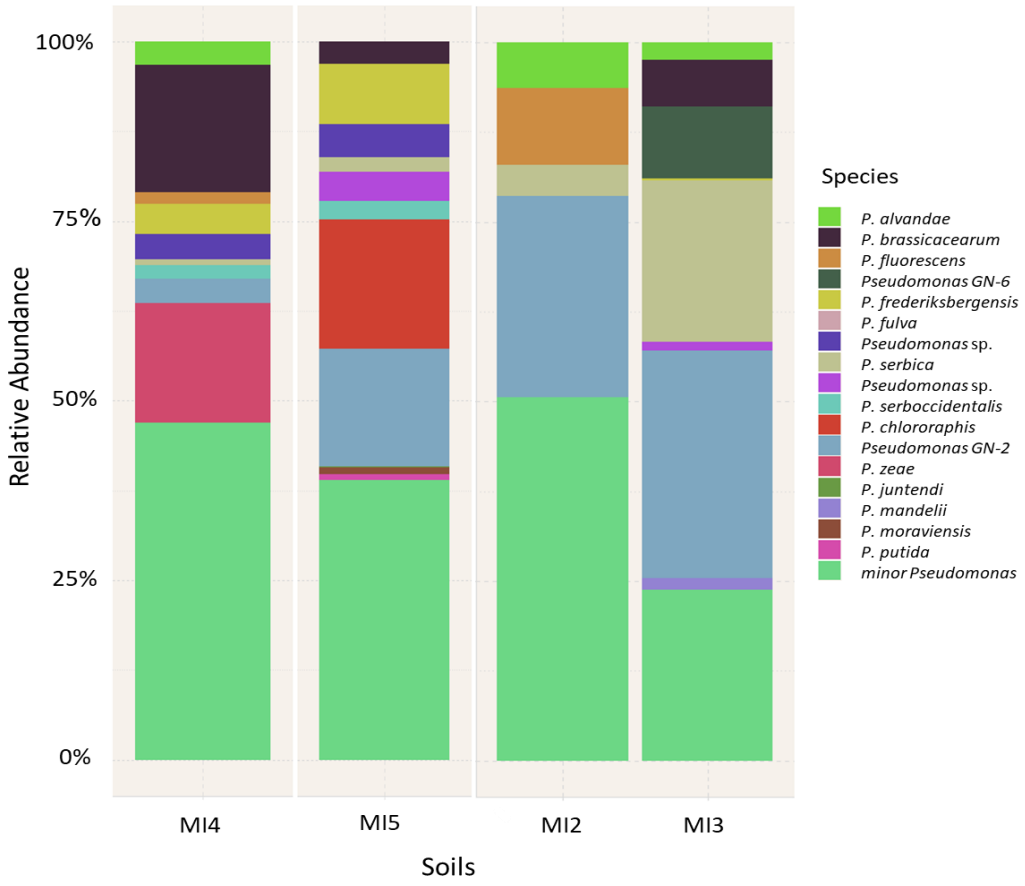

Figure 26. Relative abundance (sequence %) of different *Pseudomonas* species in soils MI4, MI5, MI2 and MI3. Species with relative abundance of < 1% were included into 'minor *Pseudomonas*'. Different *Pseudomonas* sp. represent different species based on a *rpoD* gene sequence.

The *Pseudomonas* subcommunity consisted of four to 12 species, depending on the soil, and their genotypic profiles varied from one soil to the next (Figure 26). In summary, the *Pseudomonas* subcommunity differed between the individual soils.

5.7.2. Taxonomic characterization of *Pseudomonas* isolates

A total of 406 putative *Pseudomonas* isolates were obtained from eight tested conditions - four MI soils, inoculated or not inoculated with *F. graminearum* Fg1. The taxonomic characterization of isolates using the *rpoD* primers specific for the *P. fluorescens* group was successful for 185 of them, yielding 65 different *rpoD* sequences. *rpoD*-sequenced isolates belonged to seven out of 11 subgroups of the *P. fluorescens* group that are outlined in Girard et al. (2021), specifically the subgroups *P. fluorescens*, *P. kielensis*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata* and *P. chlororaphis*, while none of the isolates belonged to the subgroups *P. protegens*, *P. asplenii*, *P. gessardii* or *P. fragi* (Supplementary material; Chapter 4B; Figure S3). *rpoD* and *rrs* gene sequences of putative fluorescent *Pseudomonas* were deposited into the EBI/EMBL database under the accession number PRJEB64203.

Gblock (Castresana, 2000; Talavera and Castresana, 2007) and seqkit (Shen et al., 2016) softwares were used to identify one isolate for each of the 65 *rpoD* sequences, and 29 of them were chosen for genome sequencing. Isolates were chosen from all four soils, i.e., eight from soil MI2, five from soil MI3, nine from soil MI4 and seven from soil MI5, from both inoculated and non-inoculated wheat, i.e., 16 from inoculated wheat and 13 from non-inoculated wheat. Phylogenetic analysis of the 29 isolates confirmed that they were phylogenetically diverse (Figure 27). The putative *Pseudomonas* isolates not amenable to *rpoD* sequencing were characterized by sequencing the 16S rRNA gene *rrs* (Supplementary material; Chapter 4B; Figure S4), yielding 52 more *Pseudomonas* isolates, however, future studies are needed in order to phylogenetically describe them at the species level.

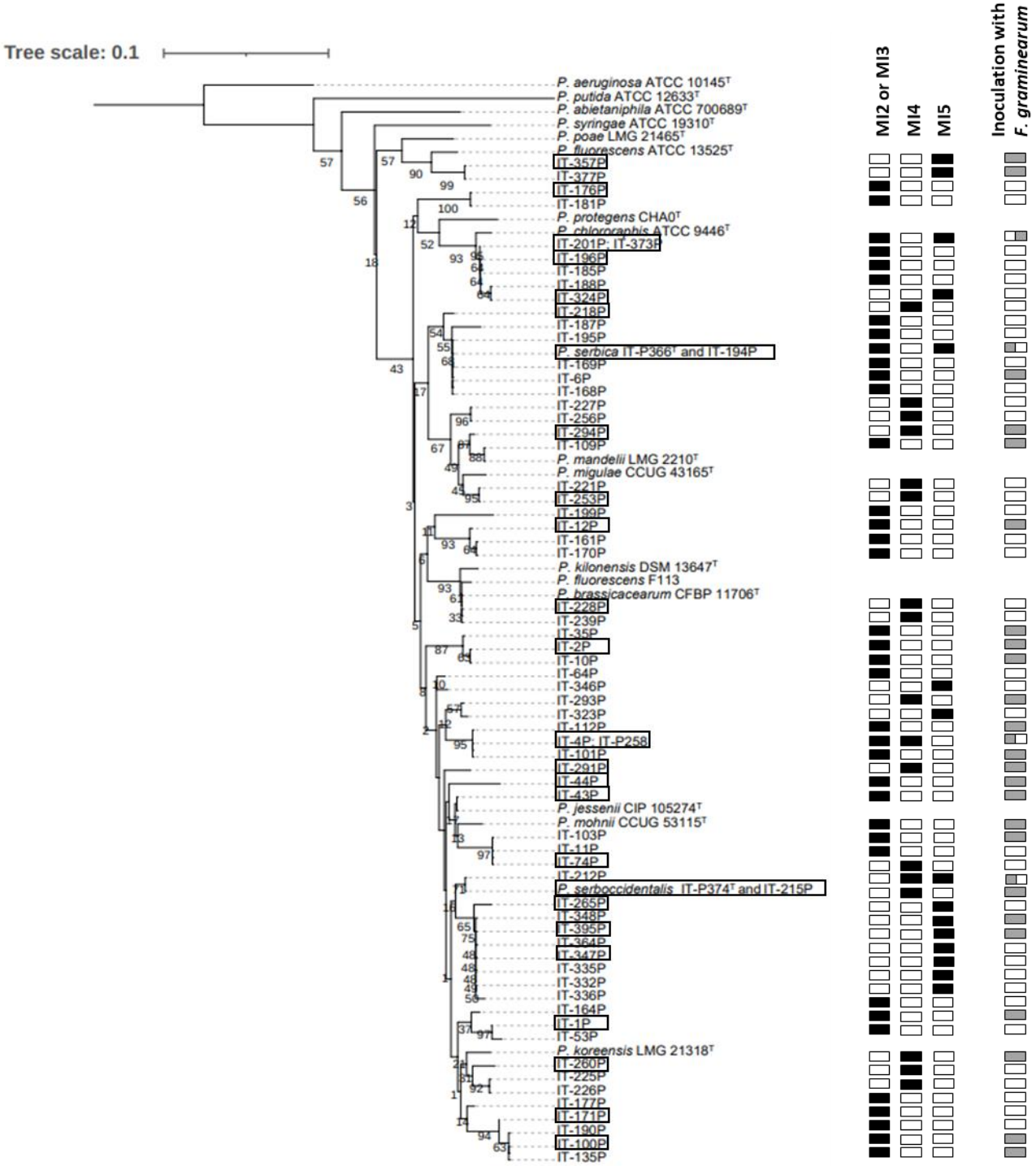

Figure 27. Phylogenetic tree of the 65 *Pseudomonas* with different *rpoD* gene sequences, including 14 *Pseudomonas* type strains (Garrido-Sanz et al., 2016) and *Pseudomonas aeruginosa* ATCC 10145^T, used for tree rooting. The tree was constructed using the SeaView multiplatform (Gouy et al., 2010), with Distance method and 1000 bootstraps, and visualized using iTol (Letunic and Bork, 2021). Strains chosen for the whole genome sequencing are framed. For each strain, the soil of origin is indicated (MI2, MI3, MI4 or MI5), and the inoculation status of wheat (gray rectangles when *Fusarium graminearum* Fg1 was used). When two sequenced isolates belonged to the same species (IT-201P and IT-373P, IT-P366 and IT-194P, IT-4P and IT-P258, IT-P374 and IT-215P), but came from different fields, this occurrence is indicated with black rectangles. When one isolate of the same species originated from non-inoculated wheat and the other from inoculated wheat, this is indicated with a rectangle half coloured in gray.

5.7.3. Identification of *Pseudomonas* isolates from soils from Mionica through genome sequencing

The genomes of the 29 genome-sequenced *Pseudomonas* isolates were assembled and it was shown that they corresponded to 29 distinct strains. Whole-genome sequences (raw and assembled) of the 29 *Pseudomonas* have been deposited into the EBI/EMBL database under the accession number PRJEB59762. Their affiliation to the *Pseudomonas* genus was confirmed by genome sequencing data. Digital DNA-DNA hybridization values (computed with GGDC 3.0 and formula 2) of the 29 sequenced strains and their closest described *Pseudomonas* type strains (available at the TYGS database) revealed 16 novel genomospecies for 20 of the strains (Table 16), and their dDDH values were below the threshold of 70%, as recommended for species delineation (Chun et al., 2018). Strains with dDDH values >70% were *P. siliginis* IT-1P, *P. jessenii* IT-43P, *P. chlororaphis* IT-196P, *P. chlororaphis* IT-201P, *P. brassicacearum* IT-228P, *P. zeae* IT-265P, *P. chlororaphis* IT-324P, *P. marginalis* IT-357P and *P. chlororaphis* IT-373P.

Table 16. Digital DNA-DNA hybridization (dDDH) values of the 20 sequenced *Pseudomonas* strains and their closest described species (available in the TYGS database), whose dDDH values are below 70%, a recommended cut-off value for bacterial species delineation. dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0) (Meier-Kolthoff and Göker, 2019; Meier-Kolthoff et al., 2022), using the recommended BLAST method, and formula 2.

Soil	Species name from TYGS	*P. glycinae* MS586 ^{T}	*P. jessenii* DSM 17150 ^{T}	*P. pisciciola* P50 ^{T}	*P. migulae* NBRC 103157 ^{T}	*P. kielensis* MBT-1 ^{T}	*P. umsongensis* DSM 16611 ^{T}	*P. koreensis* LMG 21318 ^{T}	*P. silesiensis* A3 ^{T}	*P. arsenicoxydans* CECT 7543 ^{T}	*P. tensinigenes* ZA 5.3 ^{T}	*P. neuropathica* P155 ^{T}
MI2	*Pseudomonas* sp. IT-2P	58.2										
	Pseudomonas sp. IT-4P		54.6									
	Pseudomonas sp. IT-12P			28.9								
	Pseudomonas sp. IT-44P				41.8							
	Pseudomonas sp. IT-74P		52.3									
MI3	*Pseudomonas* sp. IT-100P				50.4							
	Pseudomonas sp. IT-171P				50.9							
	Pseudomonas sp. IT-176P					57.4						
	Pseudomonas sp. IT-194P						41.1					
MI4	*Pseudomonas* sp. IT-215P							48.5				
	Pseudomonas sp. IT-218P						41.8					
	Pseudomonas sp. IT-253P								33.2			
	Pseudomonas sp. IT-P258		53.5									
	Pseudomonas sp. IT-260P							43.4				
	Pseudomonas sp. IT-291P		48.4									
	Pseudomonas sp. IT-294P									61		
MI5	*Pseudomonas* sp. IT-347P										42.8	
	Pseudomonas sp. IT-P366						41					
	Pseudomonas sp. IT-P374							48.6				
	Pseudomonas sp. IT-395P											47.3

5.7.4. Description of the new *Pseudomonas* species

Since the whole-genome sequencing data enabled uncovering 16 novel genomospecies, in the next phase of this research, two novel *Pseudomonas* species were formally described, with proposed names *P. serbica* and *P. serboccidentalis* (Oren and Goker, 2023; Todorović et al., 2023a). These two species were chosen to be described because both contained two strains that originated from different fields in Mionica, i.e., *P. serbica* contained strains IT-P366^T (= CFBP 9060^T = LMG 32732^T = EML 1791^T) and IT-194P, while *P. serboccidentalis* contained strains T-P374^T (= CFBP 9061^T = LMG 32734^T = EML 1792^T) and IT-215P (Table 16). Whole-genome sequences of type strains IT-P366^T and T-P374^T were deposited into the EBI/EMBL database, under the accession numbers PRJNA863439 and PRJNA859669, respectively.

5.7.4.1. Phylogenetic and genomic analyses of *Pseudomonas serbica* and *Pseudomonas serboccidentalis*

The phylogenetic analysis of the two novel species and construction of the phylogenetic tree inferred from *rrs* sequences (Figure 28) showed that *P. serbica* IT-P366^T and IT-194P clustered together with *P. mohnii*, while *P. serboccidentalis* IT-P374^T and IT-215P formed a cluster close to *P. gozinkensis*, *P. granadensis*, *P. monsensis*, *P. allokribbensis*, *P. glycinae*, *P. fitomaticsae* and *P. kribbensis*.

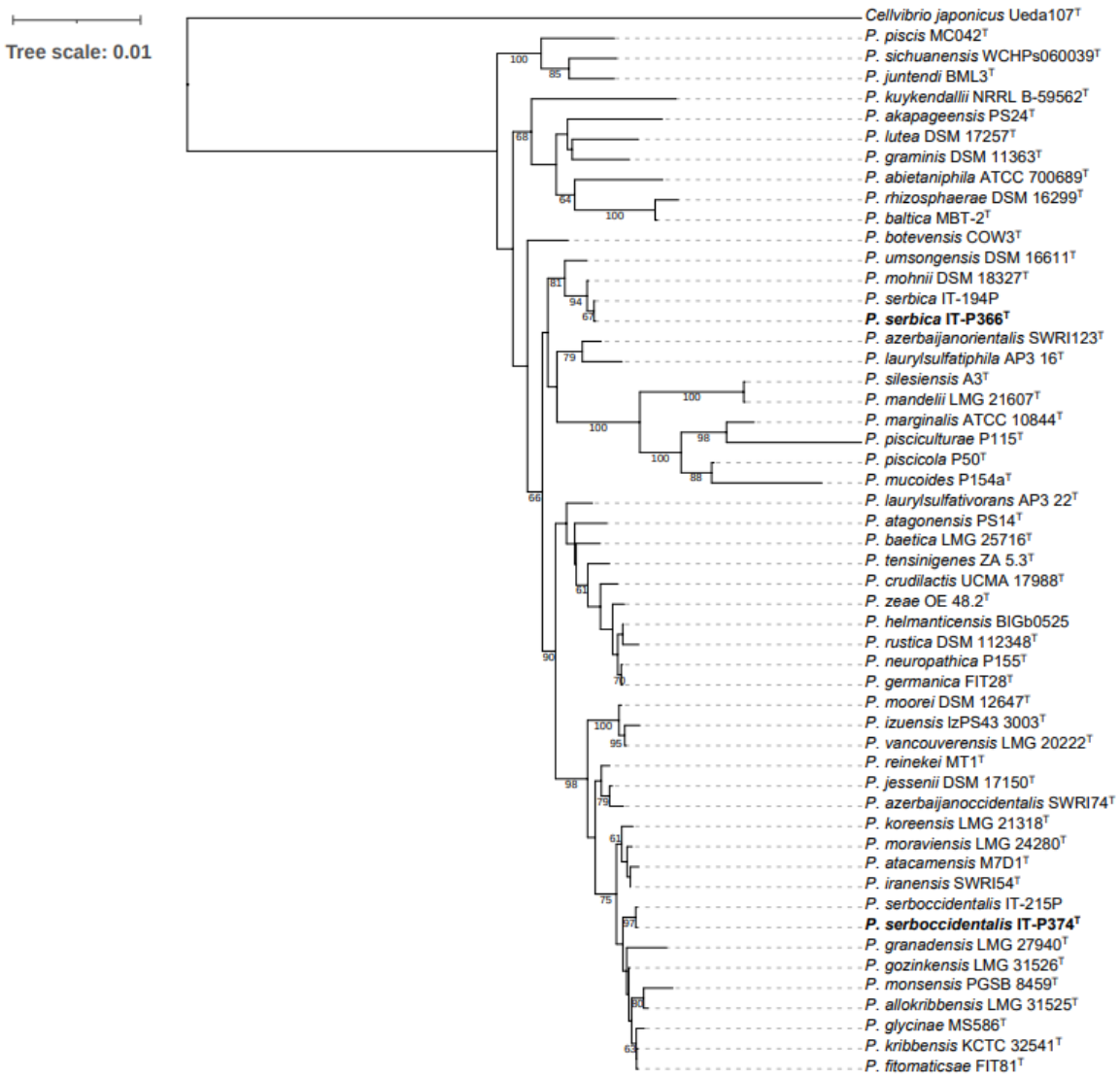

Figure 28. Phylogenetic tree of housekeeping *rrs* gene showing the relation of *Pseudomonas serbica* strains IT-P366^T (in bold) and IT-194P and *Pseudomonas serboccidentalis* strains IT-P374^T (in bold) and IT-215P with representative strains of *Pseudomonas*. The tree was constructed using TYGS server, inferred with FastME 2.1.6.1 (Lefort et al., 2015) from Genome BLAST Distance Phylogeny (GBDP) distances, calculated from *rrs* gene sequences. Numbers at the branching points are GBDP pseudo-bootstrap support values >60% from 100 replications. The tree was visualized using iTOL software (Letunic and Bork, 2021). *Cellvibrio japonicus* Ueda 107^T was used as the outgroup. Accession numbers for all the type strains used to construct the tree are given in Supplementary material; Chapter 4A; Table S4.

However, when the phylogenetic tree was inferred from whole-genome sequences (using TYGS) (Figure 29), the closest species to *P. serbica* strains IT-P366^T and IT-194P was in fact *P. umsongensis*, and the closest species to *P. serboccidentalis* strains IT-P374^T and IT-215P was *P. koreensis*. Accession numbers for all the strains used to construct the tree are given in Supplementary material; Chapter 4A; Table S4.

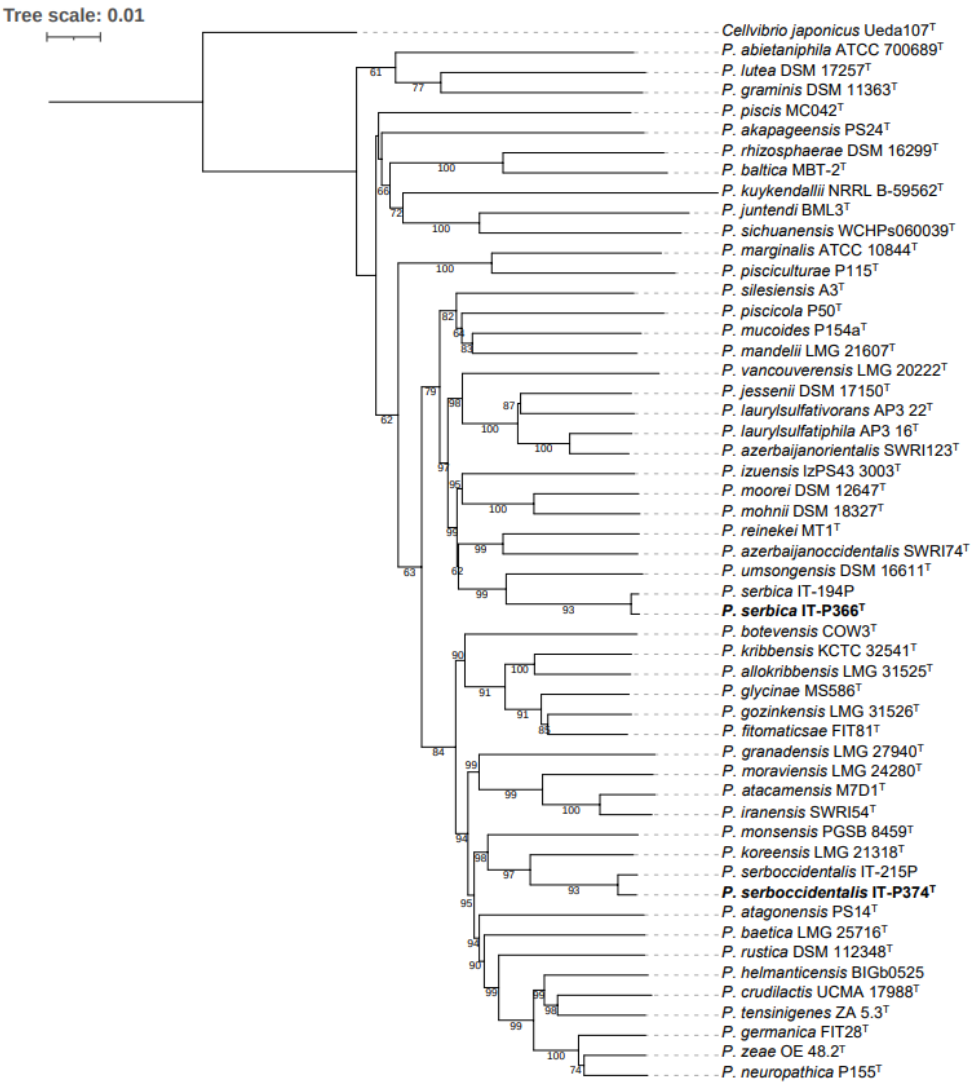

Figure 29. Phylogenetic tree using whole-genome sequences showing the relation of *Pseudomonas serbica* strains IT-P366^T (in bold) and IT-194P and *Pseudomonas serboccidentalis* strains IT-P374^T (in bold) and IT-215P with representative strains of *Pseudomonas*. The tree was constructed using TYGS server, inferred with FastME 2.1.6.1 (Lefort et al., 2015) from GBDP distances, calculated from genome sequences. Numbers at the branching points are GBDP pseudo-bootstrap support values > 60% from 100 replications. The tree was visualized using iTOL software (Letunic and Bork, 2021). *Cellvibrio japonicus* Ueda 107^T was used as the outgroup. Accession numbers for all of the type strains used to construct the tree are given in Supplementary material; Chapter 4A; Table S4.

The proposition of two new species was based on dDDH values (computed with GGDC 3.0 and formula 2) for strains IT-P366^T (proposed type strain for *P. serbica*) and IT-P374^T (proposed type strain for *P. serboccidentalis*), which were lower than the threshold of 70% when comparing with the closest type strains available in the database (Table 17 and Table 18). Furthermore, ANIb values with the closest related strains were 89.52% for strain IT-P366^T and 91.86% for strain IT-P374^T, which is below the species-delimiting threshold of 95-96%. These criteria were also passed by the strains IT-194P (proposed *P. serbica*, Table 17) and IT-215P (proposed *P. serboccidentalis*, Table 18). On the contrary, dDDH and ANIb values were 95.10 and 98.74 for strains IT-P366^T and IT-194P, respectively (within the proposed *P. serbica*), and 88.10 and 98.45 for IT-P374^T and IT-215P, respectively (within the proposed *P. serboccidentalis*), thus confirming that these pairs of strains belonged to the same two species.

Table 17. Average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values of *Pseudomonas serbica* IT-P366^T and IT-194P with the closest type strains (as seen in Figure 29). dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0) (Meier-Kolthoff and Göker, 2019; Meier-Kolthoff et al., 2022), using the recommended BLAST method. For ANIb calculations, genomes from *Pseudomonas umsongensis* DSM 16611^T, *Pseudomonas azerbaijanoccidentalis* SWRI74^T, *Pseudomonas reinekei* MT1^T, *Pseudomonas mohnii* DSM 18327^T, *Pseudomonas moorei* DSM 12647^T and *Pseudomonas izuensis* lzPS43_3003^T were available at the JSpecies server (Richter et al., 2016). Calculation of % of 16S rRNA identity of IT-P366^T and the closest type strains was done using the EzBioCloud server (Yoon et al., 2017).

	% 16S rRNA identity with type strain IT-P366^T	IT-P366^T		IT-194P	
		ANI	dDDH	ANI	dDDH
P. umsongensis DSM 16611^T	99.63	89.52	41	89.61	41.10
P. azerbaijanoccidentalis SWRI74^T	98.31	86.14	33.30	86.05	33.30
P. reinekei MT1^T	98.36	86.37	33.50	86.38	33.50
P. mohnii DSM 18327^T	100	85.93	33	85.93	33.10
P. moorei DSM 12647^T	99.81	85.92	33	85.94	33
P. izuensis lzPS43_3003^T	99	85.53[a]	33.20	85.63[a]	33.20
P. serbica IT-P366^T				98.70	95.10
P. serbica IT-194P	100	98.74	95.10		

[a] Genome coverage for ANIb calculations between each comparison was > 69%, except in the case of *P. izuensis* lzPS43_3003 and *P. serbica* strains IT-P366^T and IT-194P, where the genome coverage between *P. izuensis* lzPS43_3003 and these strains was 67.95 and 68.02, respectively.

Table 18. Average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values of *Pseudomonas serboccidentalis* IT-P374^T and IT-215P with the closest type strains (as seen in Figure 29). dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0) (Meier-Kolthoff and Göker, 2019; Meier-Kolthoff et al., 2022), using the recommended BLAST method. For ANIb calculations, genomes from *Pseudomonas koreensis* LMG21318^T and *Pseudomonas monsensis* PGSB 8459^T were available at the JSpecies server (Richter et al., 2016), and the genome coverage between each comparison was > 69%. Calculation of % of 16S rRNA identity of IT-P374^T and the closest type strains was done using the EzBioCloud server (Yoon et al., 2017).

	% 16S rRNA identity with type strain IT-P374^T	IT-P374^T		IT-215P	
		ANI	dDDH	ANI	dDDH
Pseudomonas koreensis LMG 21318^T	99.79	91.86	48.60	91.88	48.50
Pseudomonas monsensis PGSB 8459^T	99.34	88.58	38.70	88.48	38.40
P. serboccidentalis IT-P374^T				98.47	88.10
P. serboccidentalis IT-215P	100	98.45	88.10		

The main genomic features of *P. serbica* IT-P366^T and *P. serboccidentalis* IT-P374^T are their genome sizes of respectively 7602 and 5997 kb, with respectively 7592 and 5580 protein-coding genes, and a GC content of respectively 59.5% and 60.4% (Table 19). The genome size of *P. serbica* IT-P366^T is almost one Mbp above that of the closest type strain *P. umsongensis* DSM 16611^T. A megaplasmid of 1,059,298 bp identified in strain IT-P366^T is

absent from the genome of the second strain IT-194P of the proposed species *P. serbica*. The presence of this plasmid partly explains the large size difference between the genomes of the two strains (792,935 bp). In contrast, the genome sizes of *P. serboccidentalis* IT-P374^T and IT-215P are similar. The GC content is comparable in all the strains (Table 19).

Table 19. Genomic characteristics of *Pseudomonas serbica* IT-P366^T and *Pseudomonas serboccidentalis* IT-P374^T and their closest type strains. Genomic features of strains IT-P366^T and IT-P374^T were obtained from the MicroScope platform (Vallenet et al., 2020) and those from species *Pseudomonas koreensis* LMG21318^T, *Pseudomonas monsensis* PGSB 8459^T, *Pseudomonas umsongensis* DSM 16611^T, *Pseudomonas azerbaijanoccidentalis* SWRI74^T, *Pseudomonas reinekei* MT1^T, *Pseudomonas mohnii* DSM 18327^T, *Pseudomonas moorei* DSM 12647^T and *Pseudomonas izuensis* lzPS43_3003^T from the GenBank database.

Strains	GeneBank BioProject ID	Genome size (bp)	No. contigs	Plasmid (bp)	GC-content (%)	Protein-coding genes (CDS)
IT-P366^T	PRJNA863439	7,601,897	93	1,059,298	59.5	7592
IT-P374^T	PRJNA859669	5,997,322	39	0	60.4	5580
P. koreensis LMG 21318^T	PRJDB10510	6,064,848	41	0	60.5	5435
P. monsensis PGSB 8459^T	PRJNA639797	6,422,728	2	0	60	5533
P. umsongensis DSM 16611^T	PRJNA390488	6,701,403	14	0	59.7	5865
P. azerbaijanoccidentalis SWRI74^T	PRJNA639797	6,742,611	29	0	59.3	6015
P. reinekei MT1^T	PRJNA359931	6,249,573	63	0	59.1	5566
P. mohnii DSM 18327^T	PRJEB16418	6,592,588	2	0	59.6	5882
P. moorei DSM 12647^T	PRJNA563568	6,546,438	59	0	59.6	5877
P. izuensis lzPS43_3003^T	PRJNA594796	6,857,708	129	0	59.6	6093

Pan-genome analysis indicated that *P. serbica* strains IT-P366^T and IT-194P shared 5553 genes, and *P. serboccidentalis* strains IT-P374^T and IT-215P shared 5115 genes. Besides, the numbers of unique genes per strain were as follows: 1913 in IT-P366^T, 946 in IT-194P, 408 in IT-P374^T, and 602 in IT-215P.

5.7.4.2. Morphological, physiological and biochemical features of *Pseudomonas serbica* and *Pseudomonas serboccidentalis*

P. serbica and *P. serboccidentalis* species cell morphology revealed that they are Gram-negative. Their colonies appear circular, beige, and 2–3 mm in diameter after 48 h of incubation at 28°C on TSA medium. They are catalase and oxidase positive, and do not produce fluorescent pigment on King's B or PAF medium, but they produce it on PDA. All the strains show growth at 4°C, 10°C and 37°C, but not at 41°C, and they grow at pH 5 to 9, with an optimum at pH 7. All strains are strictly aerobic. All strains are motile by swimming movements, on plate with 0.3% agar. Results for API 20 NE and API ZYM strips are given in Table 20 and those for Biolog GEN III in Table 21 and in Supplementary material; Chapter 4A; Table S2, in comparison with literature data for *P. koreensis* LMG21318^T (Morimoto et al., 2020) and *P. umsongensis* DSM16611^T (Furmanczyk et al., 2018). Strains of *P. serbica* species have the ability to grow using D-mannitol, but cannot grow on pectin, D-galacturonic acid, L-galactonic acid lactone or α-hydroxybutyric acid, and in the presence of 8% NaCl, in contrary to *P. umsongensis* DSM16611^T. Strains of *P. serboccidentalis* species have the ability to grow

using sucrose, inosine or α-ketoglutaric acid, but cannot use L-histidine as a source of carbon, contrarily to *P. koreensis* LMG21318^T. All strains share features that are typical for *Pseudomonas* (Furmanczyk et al., 2018), such as the use of simple sugars (fructose and glucose), amino acids (L-alanine, L-arginine, L-aspartic acid and L-glutamic acid) and carboxylic acids (such as L-lactic acid, citric acid, L-malic acid and acetic acid) as sources of carbon. However, none of the four strains studied are able to use di-, tri- or tetrasaccharides (such as D-cellobiose, D-turanose, stachyose, D-raffinose, α-D-lactose), or D-salicin, *N*-acetyl-D-mannosamine, *N*-acetyl-neuraminic acid, D-sorbitol, D-glucose-6-phosphate, D-aspartic acid, D-lactic acid methyl ester, α-ketobutyric acid and acetoacetic acid.

Table 20. Phenotypic characteristics of *Pseudomonas serbica* and *Pseudomonas serboccidentalis*. For each species, data were obtained from the type strain and one related strain.

	P. serbica	*P. serboccidentalis*
General properties		
Fluorescence on PDA	+	+
Fluorescence on King's B agar	-	-
Fluorescence on PAF agar	-	-
Oxidase	+	+
Catalase	+	+
Enzyme activities (API ZYM)		
Alkaline phosphatase	+	-
Esterase (C 4)	+	+
Esterase Lipase (C 8)	+	+
Lipase (C 14)	-	-
Leucine arylamidase	+	+
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α-Chymotrypsin	-	-
Acid phosphatase	+	+
Naphthol-AS-BI-phosphohydrolase	+	+
α-Galactosidase	-	-
β-Galactosidase	-	-
β-Glucuronidase	-	-
α-Glucosidase	-	-
β-Glucosidase	-	-
N-Acetyl-β-glucosaminidase	-	-
α-Mannosidase	-	-
α-Fucosidase	-	-
Metabolism (API 20 NE)		
Nitrate reduction	+	-
Indole production from L-tryptophane	-	-
D-Glucose fermentation	-	-
L-Arginine dihydrolase	-	-
Urease	-	-
Esculin ferric citrate hydrolysis	-	
Gelatin hydrolysis	-	+
β-galactosidase	-	-
Growth on C sources (API 20NE)	-	-

D-Glucose assimilation	+	+
L-Arabinose assimilation	+	+
D-Mannose assimilation	+	+
D-Mannitol assimilation	+	+
N-Acetyl-glucosamine assimilation	d	+
D-Maltose assimilation	d	-
Potassium gluconate assimilation	+	+
Capric acid assimilation	+	+
Adipic acid assimilation	-	-
Malic acid assimilation	+	+
Trisodium citrate assimilation	+	+
Phenylacetic acid assimilation	+	-

-, negative; +, positive; d, depends on the tested strain

Table 21. Selected differential phenotypic characteristics of *Pseudomonas serbica* and *Pseudomonas serboccidentalis*, determined by Biolog GEN III microplates. For each species, data were obtained from the type strain and one related strain. Literature data are shown for *Pseudomonas umsongensis* DSM16611^T (Furmanczyk et al., 2018) and *Pseudomonas koreensis* LMG21318^T (Morimoto et al., 2020). A complete list of phenotypic characteristics is presented in Supplementary material; Chapter 4A; Table S2.

Biolog GEN III	*P. umsongensis* DSM16611^T	*P. serbica*	*P. koreensis* LMG21318^T	*P. serboccidentalis*
Carbon sources				
Sucrose	-	d	-	+
D-Fucose	-	d	-	w
Inosine	-	d	-	+
D-Mannitol	-	+	+	w
D-Serine	-	d	-	w
L-Histidine	+	+	+	-
Pectin	+	-	-	-
D-Galacturonic acid	+	-	-	-
L-Galactonic acid lactone	+	-	-	-
Glucuronamide	+	w	-	w
α-Ketoglutaric Acid	+	+	-	+
Tween 40	+	w	-	w
α-Hydroxybutyric acid	+	-	-	-
Other Biolog GEN III tests				
8% NaCl	+	-	-	w
Minocycline	-	d	-	+
Sodium bromate	+	d	-	w

-, negative; +, positive; d, depends on the tested strain; w, weak

Strains of *P. serbica* and *P. serboccidentalis* species are resistant to ticarcillin, ticarcillin/clavulanic acid and aztreonam (Table 22). However, for aztreonam, a difference in resistance level was observed between *P. serbica* IT-P366^T and IT-194P, strain IT-194P being fully resistant to aztreonam.

Table 22. Strains of *Pseudomonas serbica* and *Pseudomonas serboccidentalis* and their zones of inhibition (mm) when grown in the presence of 10 different antibiotics, tested using the disc diffusion method (Bauer et al., 1966).

Antibiotics (amount per disk)	*P. serbica*		*P. serboccidentalis*	
	IT-P366^T	IT-194P	IT-P374^T	IT-215P
Imipenem (10 μg)	37.8	32.8	28.3	27.4
Ticarcillin (75 μg)	0	0	0	0
Meropenem (10 μg)	40.0	36.9	30.7	31.9
Ciprofloxacin (5 μg)	38.9	40.2	32.3	31.4
Cefepime (30 μg)	32.2	32.8	24.0	24.9
Ticarcillin / clavulanic acid (75+10 μg)	0	0	0	0
Aztreonam (30 μg)	14.2	0	12.1	14.2
Levofloxacin (5 μg)	29.8	34.1	24.3	26.9
Amikacin (30 μg)	32.8	32.0	25.9	25.9
Tobramycin (10 μg)	26.2	26.8	22.6	23.0

Based on the phylogenetic, genomic and phenotypic characteristics of these two species, it was confirmed that they were indeed novel species within the genus *Pseudomonas*, for which the names *P. serbica* (with the type strain IT-P366^T) and *P. serboccidentalis* (with the type strain IT-P374^T) have been proposed (Oren and Goker, 2023; Todorović et al., 2023a). The full protologue descriptions of these novel species are presented in Tables 23 and 24.

Table 23. Protologue description of *Pseudomonas serbica* sp. nov.

Genus name	*Pseudomonas*
Species name	*Pseudomonas serbica*
Specific epithet	*serbica*
Species status	sp. nov.
Species etymology	ser'bi.ca. N.L. fem. adj. serbica, pertaining to Serbia
Nature of the type material	strain
Description of the new taxon and diagnostic traits	Gram-negative rods, non-spore-forming and motile, oxidase and catalase positive. Colonies are circular, beige coloured, with 2–3 mm in diameter after 48h of incubation at 28°C on TSA medium. Temperature range for growth is 4°C to 37°C with optimum growth at 28°C. Strictly aerobic. The pH range for growth is 5 to 9 with optimum growth at pH 7.0. Positive tests with Biolog GEN III: pH 5, pH 6, 1% NaCl, 4% NaCl, α-D-Glucose, D-Mannose, D-Fructose, 1% Sodium Lactate, Fusidic Acid, D-Serine, D-Mannitol, Glycerol, Troleandomycin, Rifamycin SV, L-Alanine, L-Arginine, L-Aspartic Acid, L-Glutamic Acid, L-Histidine, L-Pyroglutamic Acid, L-Serine, Lincomycin, Guanidine hydrochloride, Niaproof 4, D-Gluconic Acid, Mucic Acid, Quinic Acid, D-Saccharic Acid, Vancomycin, Tetrazolium Violet, Tetrazolium Blue, L-Lactic Acid, Citric Acid, α-Ketoglutaric Acid, L-Malic acid, Potassium Tellurite, γ-Amino-N-Butyric Acid, β-Hydroxybutyric Acid, Acetic Acid and Aztreonam. Weak tests with Biolog GEN III: Glucuronamide, Methyl Pyruvate, Bromosuccinic Acid, Tween 40 and Formic acid. Negative tests with Biolog GEN III: D-Cellobiose, Gentiobiose, D-Turanose, Stachyose, D-Raffinose, α-D-Lactose, D-Melibiose, β-Methyl-D-Glucoside, D-Salicin, *N*-Acetyl-D-Mannosamine, *N*-Acetyl-Neuraminic Acid, 8% NaCl, 3-Methyl glucose, L-Fucose, L-Rhamnose, D-Sorbitol, D-Arabitol, D-Glucose-6-Phosphate, D-Fructose-6-Phosphate, D-Aspartic Acid, Pectin, D-Galacturonic Acid, L-Galactonic Acid Lactone, D-Lactic Acid Methyl Ester, α-Hydroxybutyric Acid, α-Ketobutyric Acid and Acetoacetic Acid. Variable tests with Biolog GEN III: Dextrin, Maltose, D-Trehalose, Sucrose, *N*-Acetyl-D-Glucosamine, *N*-Acetyl-D-Galactosamine, D-Galactose, D-Fucose, Inosine, myo-Inositol, D-Serine, Minocycline, Gelatin, Glycyl-L-Proline, D-Glucuronic Acid, *p*-Hydroxyphenyl Acetic Acid, D-Malic acid, Nalidixic Acid, Lithium Chloride, Propionic Acid, Sodium Butyrate and Sodium Bromate.
Country of origin	Serbia
Region of origin	Mionica, Western Serbia
Date of isolation	26/10/2021
Source of isolation	Rhizosphere of wheat (*Triticum aestivum* L.)
Sampling date	9/06/2021
Latitude	44.24759 N
Longitude	20.09931 E
Altitude	189 m
16S rRNA gene accession nr.	OP021714
Genome accession number	GenBank accession number: PRJNA863439
Genome status	Incomplete
Genome size	7,601,897 bp
GC mol%	59.5%
Number of strains in study	02
Source of isolation of non-type strains	Rhizosphere of wheat (*Triticum aestivum* L.)
Information related to the Nagoya Protocol	Implementation of Nagoya Protocol is still not fully in place in Serbia, Mr. Dusan Ognjanovic (Serbian representative for agreements on biological and genetic resources) was contacted regarding this issue.
Designation of the Type Strain	IT-P366^T
Strain Collection Numbers	CFBP 9060^T, LMG 32732^T, EML 1791^T

Table 24. Protologue description of *Pseudomonas serboccidentalis* sp. nov.

Genus name	*Pseudomonas*
Species name	*Pseudomonas serboccidentalis*
Specific epithet	*serboccidentalis*
Species status	sp. nov.
Species etymology	serb.oc.ci.den.ta'lis. N.L. fem. n. Serbia, a Balkan country; L. masc. adj. occidentalis, western; N.L. fem. adj. serboccidentalis, pertaining to western Serbia
Nature of the type material	strain
Description of the new taxon and diagnostic traits	Gram-negative rods, non-spore-forming and motile, oxidase and catalase positive. Colonies are circular, beige coloured, with 2–3 mm in diameter after 48h of incubation at 28 °C on TSA medium. Temperature range for growth is 4 °C to 37 °C with optimum growth at 28 °C. Strictly aerobic. The pH range for growth is 5 to 9 with optimum growth at pH 7.0. Positive tests with Biolog GEN III: Sucrose, pH 5, pH 6, 1% NaCl, 4% NaCl, α-D-Glucose, D-Mannose, D-Galactose, Inosine, 1% Sodium Lactate, Fusidic Acid, D-Serine, Troleandomycin, Rifamycin SV, Minocycline, L-Alanine, L-Arginine, L-Aspartic Acid, L-Glutamic Acid, L-Pyroglutamic Acid, Lincomycin, Guanidine hydrochloride, Niaproof 4, D-Gluconic Acid, Mucic Acid, Quinic Acid, D-Saccharic Acid, Vancomycin, Tetrazolium Violet, Tetrazolium Blue, L-Lactic Acid, Citric Acid, α-Ketoglutaric Acid, L-Malic Acid, Nalidixic Acid, Lithium Chloride, Potassium Tellurite, γ-Amino-N-Butyric Acid, β-Hydroxybutyric Acid, Propionic Acid, Acetic Acid and Aztreonam. Weak tests with Biolog GEN III: *N*-Acetyl-D-Glucosamine, 8% NaCl, D-Fructose, D-Fucose, D-Mannitol, D-Serine, L-Serine, Glucuronamide, Sodium Bromate and Tween 40. Negative tests with Biolog GEN III: Dextrin, Maltose, D-Trehalose, D-Cellobiose, Gentiobiose, D-Turanose, Stachyose, D-Raffinose, α-D-Lactose, D-Melibiose, β-Methyl-D-Glucoside, D-Salicin, *N*-Acetyl-D-Mannosamine, *N*-Acetyl-D-Galactosamine, *N*-Acetyl-Neuraminic Acid, 3-Methyl glucose, L-Fucose, L-Rhamnose, D-Sorbitol, D-Arabitol, myo-Inositol, D-Glucose-6-Phosphate, D-Fructose-6-Phosphate, D-Aspartic Acid, Gelatin, Glycl-L-Proline, L-Histidine, Pectin, D-Galacturonic Acid, L-Galactonic Acid Lactone, D-Glucuronic Acid, *p*-Hydroxyphenyl Acetic Acid, Methyl Pyruvate, D-Lactic Acid Methyl Ester, D-Malic Acid, Bromosuccinic Acid, α-Hydroxybutyric Acid, α-Ketobutyric Acid, Acetoacetic Acid, Formic Acid and Sodium Butyrate.
Country of origin	Serbia
Region of origin	Mionica, Western Serbia
Date of isolation	26/10/2021
Source of isolation	Rhizosphere of wheat (*Triticum aestivum* L.)
Sampling date	9/06/2021
Latitude	44.24759 N
Longitude	20.09931 E
Altitude	189 m
16S rRNA gene accession nr.	OP021715
Genome accession number	GenBank accession number: PRJNA859669
Genome status	Incomplete
Genome size	5,997,322 bp
GC mol%	60.4%
Number of strains in study	02
Source of isolation of non-type strains	Rhizosphere of wheat (*Triticum aestivum* L.)
Information related to the Nagoya Protocol	Implementation of Nagoya Protocol is still not fully in place in Serbia, Mr. Dusan Ognjanovic (Serbian representative for agreements on biological and genetic resources) was contacted regarding this issue.
Designation of the Type Strain	IT-P374^T
Strain Collection Numbers	CFBP 9061^T, LMG 32734^T, EML 1792^T

5.7.5. Distribution of sequenced *Pseudomonas* strains in soils

After formally describing two novel *Pseudomonas* species, the study was continued to achieve the third objective of this research, which was to identify the genomic and functional particularities of *Pseudomonas* bacteria isolated from suppressive vs. non-suppressive soils. Different *Pseudomonas* taxa were evidenced in different soils when considering the 29 sequenced *Pseudomonas* strains. These *Pseudomonas* belonged to the seven subgroups (i.e., *P. fluorescens*, *P. kielensis*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata* and *P. chlororaphis* subgroups) of the *P. fluorescens* group (Figure 30). Besides two novel genomospecies formally described and named *P. serbica* and *P. serboccidentalis*, dDDH hybridization values (computed with GGDC 3.0 and formula 2) of the 29 sequenced strains and their closest described *Pseudomonas* type strains (available at the TYGS database) revealed 14 additional novel genomospecies (hereafter termed *GN-1* to *GN-14*) for 16 of the strains, and their dDDH values were below the threshold of 70% (Table 16). From MI2 soil, five novel genomospecies (*GN-1* to *GN-5*) were found, together with one *P. siliginis* and one *P. jessenii* strain. From MI3 soil, two novel genomospecies were obtained (*GN-6* and *GN-7*), together with one *P. serbica* and two *P. chlororaphis* strains. From MI4 soil, six novel genomospecies (*GN-2*, also present in MI2, and *GN-8 to GN-12*) were found, one *P. zeae*, one *P. brassicacearum* and one *P. serboccidentalis* strain. From MI5 soil, two novel genomospecies (*GN-13* and *GN-14*) were evidenced, along with one *P. marginalis*, one *P. serbica*, one *P. serboccidentalis* and two *P. chlororaphis* strains.

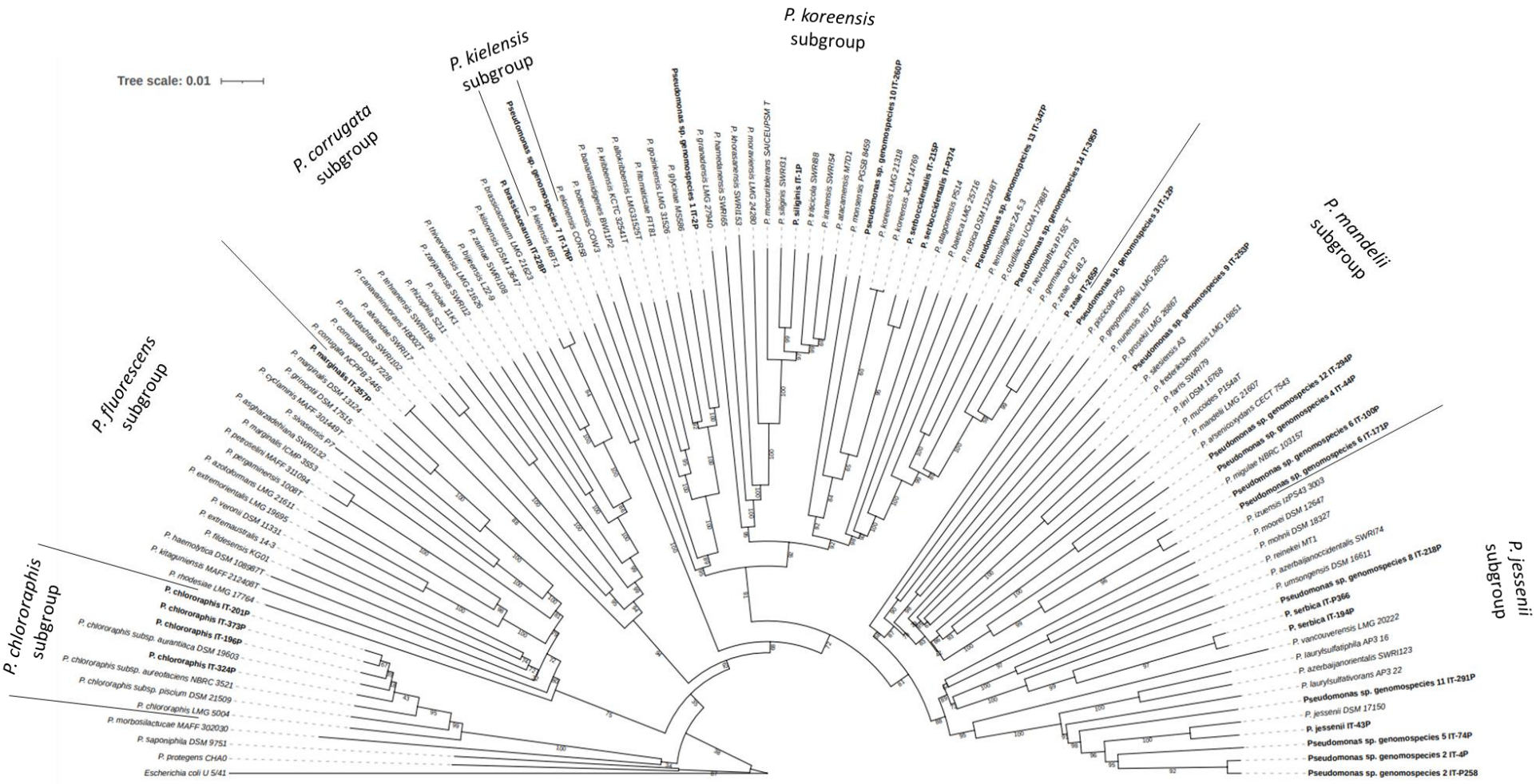

Figure 30. Phylogenetic tree of 29 *Pseudomonas* strains (in bold) whose genomes were sequenced, including *Pseudomonas* type strains (Garrido-Sanz et al., 2016) from the TYGS database and *E. coli* U 5/41^{T}, used for tree rooting. The tree was constructed using TYGS server, inferred with FastME 2.1.6.1 (Lefort et al., 2015) from GBDP distances, calculated from genome sequences. Numbers at the branching points are GBDP pseudo-bootstrap support values from 100 replications. The tree was visualized using iTOL software (Letunic and Bork, 2021).

5.7.6. Genomic comparison of sequenced *Pseudomonas* strains

In the *P. chlororaphis* subgroup (Figure 30), the four *P. chlororaphis* strains IT-196P, IT-201P (from the soil MI3), IT-324P and IT-373P (from the soil MI5) had a genome size ranging from 6,532 to 7,133 kb, with 6260 to 6872 coding DNA sequences (CDS) and GC content from 62.78% to 63.09% (Table 25). In the *P. koreensis* subgroup, genome sizes of *P. serboccidentalis* IT-215P (from the soil MI4) and IT-P374 (from the soil MI5) were 6,124 kb and 5,997 kb, with 5777 and 5582 CDS and GC contents of 60.29% and 60.36%, respectively. Other strains from the *P. koreensis* subgroup, i.e., *Pseudomonas GN-1* IT-2P, *GN-10* IT-260P, *GN-13* IT-347P, *GN-14* IT-395P, *P. zeae* IT-265P and *P. siliginis* IT-1P had genome size ranging from 5,841 kb to 6,699 kb, 5415 to 6303 CDS, and GC content between 59.10% and 60.51%. The only representative of the *P. kielensis* subgroup, *Pseudomonas GN-7* IT-176P, had genome size of 5,962 kb, 5602 CDS and GC content of 61.20%. In the *P. jesseni* subgroup, *P. serbica* IT-P366 from MI5 soil possessed a larger genome (7,602 kb) than that of *P. serbica* IT-194P from MI3 soil (6,942 kb), due to the presence of a 1,059,298-bp megaplasmid in the former, as well as a large number of CDS (7598 and 6770 CDS, respectively). *P. serbica* IT-P366 had a GC content of 59.55%, while the GC content in *P. serbica* IT-194P was 58.81%. *Pseudomonas* sp. IT-4P from soil MI2 and IT-P258 from soil MI4 (belonging to genomospecies *GN-2* within the *P. jessenii* subgroup) had genome size of 6,312 kb and 6,283 kb, with 5997 and 5915 CDS, and GC content of 59.94% and 59.95%, respectively. Strain IT-4P also contained a 9,290-bp plasmid. *Pseudomonas GN-5* IT-74P, *GN-11* IT-291P and *GN-8* IT-218P, and *P. jessenii* IT-43P had comparable genome sizes (6,304-6,581 kb), CDS (6057-6319) and GC contents (59.61-60.58%). In the *P. mandelii* subgroup, *Pseudomonas GN-6* IT-100P and IT-171P (both from soil MI3) had similar genome sizes (respectively 6,558 and 6,551 kb), CDS (respectively 6313 and 6272) and GC contents (respectively 59.33% and 59.32%). *Pseudomonas GN-3* IT-12P, *GN-9* IT-253P, *GN-12* IT-294P and *GN-4* IT-44P had a genome size between 6,037 and 6,827 kb, with 5799 to 6487 CDS, and a GC content between 58.53% and 61.43%. In the *P. corrugata* subgroup, *P. brassicacearum* IT-228P had a genome size of 6,701 kb, with 6361 CDS and GC content of 60.90%. In the *P. fluorescens* subgroup, *P. marginalis* IT-357P had a genome of 6,611 kb, with 6259 CDS and 61.36% GC content.

When considering soils of origin, genome size was 5,841-6,943 kb for the 13 strains from soils MI2 and MI3 (*P. siliginis* IT-1P, *Pseudomonas GN-1* IT-2P, *Pseudomonas GN-2* IT-4P, *Pseudomonas GN-3* IT-12P, *P. jessenii* IT-43P, *Pseudomonas GN-4* IT-44P, *Pseudomonas GN-5* IT-74P, *Pseudomonas GN-6* strains IT-100P and IT-171P, *Pseudomonas GN-7* IT-176P*, P. serbica* IT-194P, *P. chlororaphis* IT-196P and *P. chlororaphis* IT-201P), 6,023-6,827 kb for the nine strains from soil MI4 (*P. serboccidentalis* IT-215P, *Pseudomonas GN-8* IT-218P, *P. brassicacearum* IT-228P, *Pseudomonas GN-9* IT-253P, *Pseudomonas GN-2* IT-P258, *Pseudomonas GN-10* IT-260P, *P. zeae* IT-265P, *Pseudomonas GN-11* IT-291P and *Pseudomonas GN-12* IT-294P), and 5,997-7,602 kb for the seven strains from soil MI5 (*P. chlororaphis* IT-324P, *Pseudomonas GN-13* IT-347P, *P. marginalis* IT-357P, *P. serbica* IT-P366, *P. chlororaphis* IT-373P, *P. serboccidentalis* IT-P374 and *Pseudomonas GN-14* IT-395P) (Table 25). GC content was 59.32-63.09% for the 13 strains from soils MI2 and MI3, 58.53-60.90% for the nine strains from soil MI4 and 59.19-62.99% for the seven strains from soil MI5. In summary, genome size and GC content of the 29 sequenced strains depended on the species or subgroup, regardless of the soil of origin.

Table 25. Genomic features of sequenced *Pseudomonas,* retrieved from TYGS.

Soil	Species name from TYGS	Isolate name	Genome size (bp)	Plasmid	GC content (%)	No. contigs	Coding DNA sequences (CDS)
MI2	*P. siliginis*	IT-1P	5,841,413	-	60.07	37	5415
	Pseudomonas GN-1	IT-2P	6,478,735	-	60.51	49	6041
	Pseudomonas GN-2	IT-4P	6,312,045	+	59.94	111	5997
	Pseudomonas GN-3	IT-12P	6,341,720	-	61.43	28	6001
	P. jessenii	IT-43P	6,413,346	-	59.66	75	6124
	Pseudomonas GN-4	IT-44P	6,569,010	-	59.57	101	6263
	Pseudomonas GN-5	IT-74P	6,304,484	-	59.87	93	6057
MI3	*Pseudomonas GN-6*	IT-100P	6,558,007	-	59.33	59	6313
	Pseudomonas GN-6	IT-171P	6,551,484	-	59.32	60	6272
	Pseudomonas GN-7	IT-176P	5,962,660	-	61.20	123	5602
	P. serbica	IT-194P	6,808,962	-	59.81	94	6770
	P. chlororaphis	IT-196P	6,635,492	-	63.09	29	6284
	P. chlororaphis	IT-201P	6,532,202	-	62.84	23	6260
MI4	*P. serboccidentalis*	IT-215P	6,124,801	-	60.29	67	5777
	Pseudomonas GN-8	IT-218P	6,581,279	-	60.58	96	6319
	P. brassicacearum	IT-228P	6,701,129	-	60.90	74	6361
	Pseudomonas GN-9	IT-253P	6,037,596	-	58.53	84	5799
	Pseudomonas GN-2	IT-P258	6,283,203	-	59.95	109	5915
	Pseudomonas GN-10	IT-260P	6,023,190	-	60.33	44	5566
	P. zeae	IT-265P	6,699,764	-	59.10	126	6303
	Pseudomonas GN-11	IT-291P	6,322,035	-	59.61	103	6098
	Pseudomonas GN-12	IT-294P	6,827,290	-	58.98	59	6487
MI5	*P. chlororaphis*	IT-324P	7,133,109	-	62.78	56	6872
	Pseudomonas GN-13	IT-347P	6,284,985	-	59.46	60	5743
	P. marginalis	IT-357P	6,611,256	-	61.36	47	6259
	P. serbica	IT-P366	7,601,897	+	59.55	93	7598
	P. chlororaphis	IT-373P	6,801,379	-	62.99	16	6486
	P. serboccidentalis	IT-P374	5,997,322	-	60.36	39	5582
	Pseudomonas GN-14	IT-395P	6,472,514	-	59.19	61	5965

5.7.7. Presence of genes involved in biocontrol or plant growth promotion

After the genome sequencing and assembling, genomes of the 29 *Pseudomonas* were annotated. BLAST revealed the presence of genes for biosynthesis of antimicrobial compounds in most of the 29 *Pseudomonas* strains sequenced (Table 26).

Table 26. Distribution of genes involved in biocontrol and plant-growth promotion in *Pseudomonas* strains. Presence of the property (the whole gene cluster) is marked with +, and when for certain property there are several possible pathways to achieve a function, names of the genes found in the genome are indicated. Genes were found with DIAMOND blastp (v.2.0.8.146; Buchfink et al., 2015), using the options --query-cover 80 --id 70 (query coverage >80%; amino acid identity >70%), if not specified otherwise. Origin of each strain is indicated based on soil (MI2, MI3, MI4 or MI5) and inoculation status of wheat used for isolation (i for inoculation with *Fusarium graminearum* Fg1 and c for non-inoculated wheat).

Soil	Fg1 inoculation	Isolate name	Phenazine production	HPR production	2,4-DAPG production	Pyrrolnitrin production	HCN production	Pyoverdine production	Ethylene production	ACC deaminase	Auxin biosynthesis	2,3-butanediol conversion to acetoin	Acetoin catabolism	Phosphate solubilization	Denitrification	Alkaline metalloproteinase production	FitD production	Number of phytobenefical functions
MI2	i	*P. siliginis* IT-1P					+		+					*gcd, gad*		+		4
	i	*Pseudomonas GN-1* IT-2P					+	+	+					*gcd, gad*		+		5
	i	*Pseudomonas GN-2* IT-4P						+	+		*iaaMH**			*gcd*				4
	i	*Pseudomonas GN-3* IT-12P								+	*iaaMH**				*nirS*			3
	i	*P. jessenii* IT-43P						+	+		*iaaMH**			*gcd*				4
	i	*Pseudomonas GN-4* IT-44P						+	+	+	*iaaMH**				*nirS*	+		6
	c	*Pseudomonas GN-5* IT-74P						+	+					*gcd*		+		4
MI3	i	*Pseudomonas GN-6* IT-100P						+		+					*nirS*	+		4
	c	*Pseudomonas GN-6* IT-171P						+		+					*nirS*	+		4

	c	*Pseudomonas GN-7* IT-176P									*adh*	+	*gcd*					3
	c	*P. serbica* IT-194P					+	+		*iaaMH**								3
	c	*P. chlororaphis* IT-196P	+	+		+	+	+	+		*iaaMH*	*bdhA, adh*	+	*gcd, gad*	*nirK*	+		12
	c	*P. chlororaphis* IT-201P	+			+	+	+	+		*iaaMH*	*bdhA, adh*	+	*gcd, gad*	*nirK*	+		11
MI4	c	*P. serboccidentalis* IT-215P					+	+						*gcd, gad*		+		4
	c	*Pseudomonas GN-8* IT-218P						+	+					*gad*				3
	c	*P. brassicacearum* IT-228P			+		+			+	*iaaMH**	*adh*	+		*nirS*	+		8
	c	*Pseudomonas GN-9* IT-253P																0
	c	*Pseudomonas GN-2* IT-P258							+					*gcd*				2
	i	*Pseudomonas GN-10* IT-260P					+	+	+					*gcd, gad*				4
	i	*P. zeae* IT-265P					+	+	+					*gcd, gad*		+		5
	i	*Pseudomonas GN-11* IT-291P										*adh*		*gcd*				2
	i	*Pseudomonas GN-12* IT-294P												*gad*	*nirS*	+		3
MI5	c	*P. chlororaphis* IT-324P	+	+		+	+	+	+		*iaaMH*	*bdhA, adh*	+	*gcd, gad*	*nirK*	+	+	13
	c	*Pseudomonas GN-13* IT-347P					+	+	+					*gcd, gad*		+		5
	i	*P. marginalis* IT-357P								+		*adh*	+	*gad*	*nirS*	+		6
	i	*P. serbica* IT-P366							+		*iaaMH**							2
	i	*P. chlororaphis* IT-373P	+	+		+	+	+	+		*iaaMH*	*bdhA, adh*	+	*gcd, gad*	*nirK*	+		12
	i	*P. serboccidentalis* IT-P374					+	+						*gcd, gad*		+		4
	i	*Pseudomonas GN-14* IT-395P					+	+	+					*gcd, gad*		+		5

Genes (and functions) that were searched for in the 29 *Pseudomonas* isolates, but were not found: *pltABCDEFGLM* (production of pyoluteorin), *pchABCDEF* (production of pyochelin), *pmsABCE* (production of pseudomonine), *iacABCDEFGHI* (auxin catabolism), *budB*/*ilvNB*/*alsS*, *budA*/*alsD* (acetoin biosynthesis), *budC*/*ydjL* (2,3-butanediol biosynthesis) and *nifHDK* (nitrogen fixation).
* *iaaH* found with only 30-40 % identity

In the *P. chlororaphis* subgroup (Figure 30, Table 26), the four *P. chlororaphis* strains, i.e., IT-196P, IT-201P (from the soil MI3), IT-324P and IT-373P (from the soil MI5), harbored genes involved in the production of phenazine, pyrrolnitrin, HCN, pyoverdine, ethylene, auxin, 2,3-butanediol conversion to acetoin, acetoin catabolism, phosphate solubilization, denitrification and *aprA* genes for production of alkaline metalloproteinase. All *P. chlororaphis* strains harbored genes for HPR production but strain IT-201P lacked *darC*. Additionally, *P. chlororaphis* IT-324P had the *fitD* insect-toxin gene, involved in control of insect pests. In the *P. koreensis* subgroup (Figure 30, Table 26), *P. serboccidentalis* strains IT-215P (from the soil MI4) and IT-P374 (from the soil MI5) harbored genes for the production of HCN, pyoverdine, alkaline metalloproteinase and *gcd*/*gad* genes for phosphate solubilization. Strains *Pseudomonas GN-1* IT-2P (from MI2), *GN-10* IT-260P (from MI4), *GN-13* IT-347P (from MI5), *GN-14* IT-395P (from MI5), *P. zeae* IT-265P (from MI4) and *P. siliginis* IT-1P (from MI2) harbored *gcd*/*gad* genes for phosphate solubilization. These strains contained genes for HCN, pyoverdine and ethylene production (except *P. siliginis* IT-1P, which harbored only genes for HCN and ethylene production). Gene *aprA* for production of alkaline metalloproteinase was harbored by *Pseudomonas GN-1* IT-2P, *GN-13* IT-347P, *GN-14* IT-395P, *P. zeae* IT-265P and *P. siliginis* IT-1P. In the *P. kielensis* subgroup (Figure 30, Table 26), *Pseudomonas GN-7* IT-176P (from MI3) possessed genes for 2,3-butanediol conversion to acetoin, for acetoin catabolism and *gcd* gene for phosphate solubilization. In the *P. jessenii* subgroup (Figure 30, Table 26), *P. serbica* IT-P366 (from MI5 soil) and IT-194P (from MI3 soil) possessed genes involved in the modulation of plant hormonal levels, i.e., auxin biosynthesis and ethylene production. Moreover, *P. serbica* IT-194P harbored *pvdL* gene for pyoverdine production. *Pseudomonas GN-2* strains IT-4P (from soil MI2) and IT-P258 (from soil MI4) harbored *gcd* (phosphate solubilization) and *efe* (ethylene production), and strain IT-4P has the potential of producing pyoverdine and auxin. *Pseudomonas GN-5* IT-74P (from MI2), *GN-11* IT-291P (from MI4) and *GN-8* IT-218P (from MI4), and *P. jessenii* IT-43P (from MI2) harbored *gcd* and all four but IT-291P possessed genes for pyoverdine and ethylene production. Moreover, IT-43P contained genes for auxin biosynthesis, IT-74P displayed genes for alkaline metalloproteinase production and IT-291P contained genes for 2,3-butanediol conversion to acetoin. In the *P. mandelii* subgroup (Figure 30, Table 26), *Pseudomonas GN-6* IT-100P and IT-171P (both from soil MI3) exhibited the potential of producing pyoverdine, ACC deaminase, alkaline metalloproteinase and for denitrification. *Pseudomonas GN-3* IT-12P (MI2 soil) had *nirS* and genes for ACC deaminase and auxin production. *Pseudomonas GN-4* strain IT-44P, also from the MI2 soil, harbored the same three genes but also genes for pyoverdine, ethylene and alkaline metalloproteinase production. *Pseudomonas GN-12* IT-294P (soil MI4) had genes for P solubilization, denitrification and alkaline metalloproteinase production. *Pseudomonas GN-9* IT-253P (from MI4) harbored none of the genes investigated. In the *P. corrugata* subgroup (Figure 30, Table 26), *P. brassicacearum* IT-228P (from soil MI4) was the only one harboring gene for DAPG production, besides genes for HCN, ACC deaminase, auxin and alkaline metalloproteinase production, and genes for 2,3-butanediol conversion to acetoin, acetoin catabolism and denitrification. Finally, in the *P. fluorescens* subgroup (Figure 30, Table 26), *P. marginalis* IT-357P (from soil MI5) had genes for ethylene and alkaline metalloproteinase production, phosphate solubilization, denitrification, and genes for 2,3-butanediol conversion to acetoin and acetoin catabolism.

Altogether, HCN genes were the most common (in 13 strains from all soils), followed by those for pyrrolnitrin and phenazine (each present in four strains from soils MI2, MI3 and MI5), HPR (three strains from soils MI2, MI3 and MI5) and DAPG (one MI4 strain). None had pyoluteorin genes. Many strains (18 of 29, from all soils) also had an *aprA* protease gene and one

MI5 strain had the *fitD* insect-toxin gene. The pyoverdine gene *pvdL* was found in 19 strains (from all soils), and all genomes shared partial homologies with siderophore BGCs known from *Pseudomonas* (pyoverdine) as well as non-*Pseudomonas* bacteria. Genes for secondary siderophores pyochelin and pseudomonine were not detected. Most of the strains (from all soils) had the potential for interfering with plant hormonal levels, i.e., 18 harbored *efe*, six possessed *acdS*, 11 displayed *iaaM* and *iaaH* (though seven strains had only 30-40% identity with the query *iaaH* from *Pseudomonas* JV395A), but none had auxin catabolism genes. Eight strains (from all soils) had *adh* and four of them also had *bdhA*, but none of them harbored genes for acetoin or 2,3-butanediol synthesis. In addition, seven strains (from all soils) had *aco* genes for acetoin catabolism. Many strains (from all soils) displayed genes influencing plant nutritional status, via phosphate solubilization (*gcd* and/or *gad* in 21 strains) and denitrification (*nirK*/*nirS* in 11 strains), while *nifHDK* were not found.

Annotation of CAZymes showed that in the *P. chlororaphis* subgroup, the four *P. chlororaphis* strains, i.e., IT-196P, IT-201P (from the soil MI3), IT-324P and IT-373P (from the soil MI5), harbored genes encoding chitinases and betaglucanases (except IT-201P) (Figure 32), and genes of the AA10 family (which includes lytic polysaccharide monooxygenases that potentially target chitin) (Figure 31), but none of them harbored genes that code for cellulases and mannanases (Figure 32). In the *P. koreensis* subgroup, *P. serboccidentalis* strains IT-215P (from the soil MI4) and IT-P374 (from the soil MI5) contained genes involved in the production of chitinases and genes of the AA10 family. Strains *Pseudomonas GN-1* IT-2P (from MI2), *GN-10* IT-260P (from MI4), *GN-13* IT-347P (from MI5), *GN-14* IT-395P (from MI5), *P. zeae* IT-265P (from MI4) and *P. siliginis* IT-1P (from MI2) also had the potential of producing chitinases and lytic polysaccharide monooxygenases, and some of them (IT-1P, IT-2P and IT-347P) also had the potential of producing betaglucanases. In the *P. kielensis* subgroup, *Pseudomonas GN-7* IT-176P (from MI3) could potentially produce chitinases and betaglucanases, as shown by the annotation of CAZymes. In the *P. jessenii* subgroup, both *P. serbica* IT-P366 (from MI5 soil) and IT-194P (from MI3) contained genes for chitinases and betaglucanases production, and genes of the AA10 family, but only IT-194P possessed genes for cellulase production. *Pseudomonas GN-2* strains IT-4P (from soil MI2) and IT-P258 (from soil MI4) harbored genes of the AA10 family, and IT-P258 contained chitinases genes. *Pseudomonas* strains IT-218P and IT-291P (both from MI4) had genes for cellulases, IT-74P (from MI2) and IT-218P had genes that code for chitinases, IT-43P (from MI2) and IT-218P had genes encoding betaglucanases, while all but IT-74P had genes of the AA10 family. In the *P. mandelii* subgroup, *Pseudomonas GN-6* IT-100P and IT-171P (both from soil MI3) had genes encoding chitinases and betaglucanases, as well as genes of the AA10 family. Inspection of CAZymes showed that the four strains, i.e., *Pseudomonas GN-3* IT-12P (MI2 soil), *Pseudomonas GN-12* IT-294P (soil MI4), *Pseudomonas GN-9* IT-253P (soil MI4) and *Pseudomonas GN-4* IT-44P (soil MI2), harbored genes for betaglucanases, all but IT-253P harbored genes of the AA10 family and chitinases, and only IT-44P and IT-253P harbored cellulases genes. In the *P. corrugata* subgroup, *P. brassicacearum* IT-228P (from soil MI4) has the potential of producing chitinases. Finally, in the *P. fluorescens* subgroup, *P. marginalis* IT-357P (from soil MI5) had genes for the production of chitinases, cellulases and genes of the AA10 family.

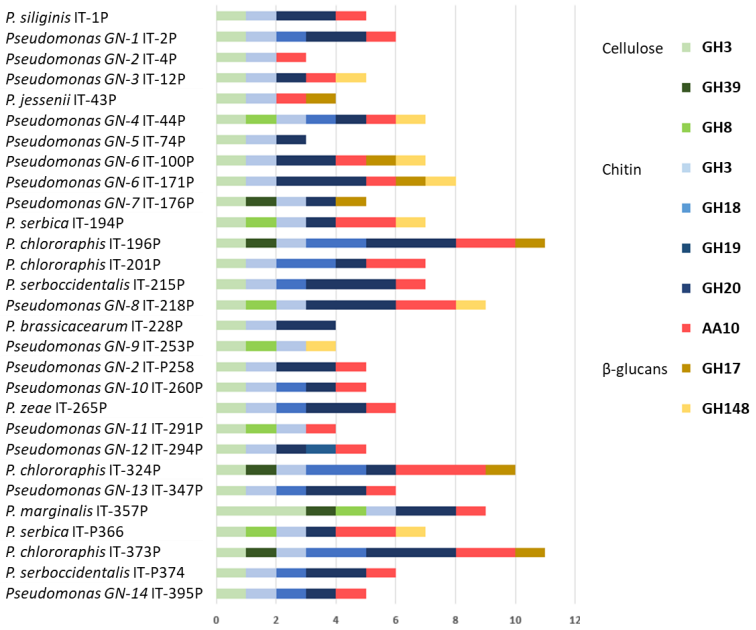

Figure 31. Abundance of genes belonging to CAZyme families potentially targeting cell wall components in fungi and oomycetes (cellulose, chitin and β-glucans), found in the genomes of sequenced *Pseudomonas* isolates.

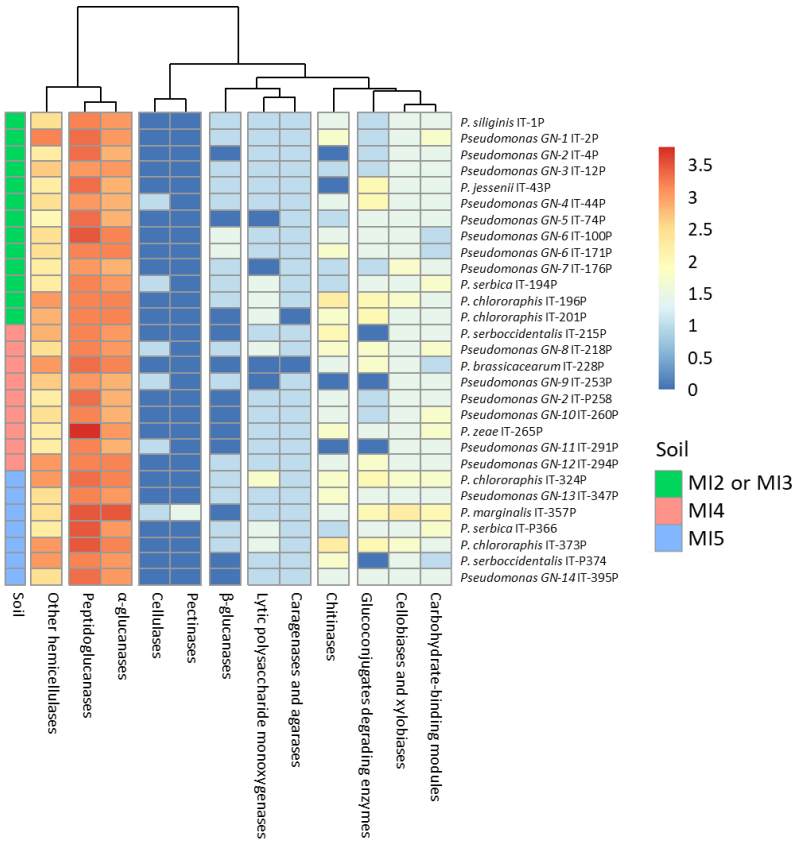

Figure 32. Heatmap showing the abundance of CAZyme genes annotated for each function found in the genomes of the 29 *Pseudomonas*. Legend shows transformed counts.

In summary, genes involved in phytobeneficial functions were spread quite evenly among *Pseudomonas* strains regardless of the experimental conditions (field of origin, suppressiveness status, previous manure application; Table 26). Yet, the biosynthetic genes for antimicrobial compounds phenazine, HPR and pyrrolnitrin were restricted to four *P. chlororaphis* strains from MI3 (manure used; fungistatic and suppressive) or MI5 (no manure; non-fungistatic and suppressive) soils. *Pseudomonas* strains from all four soils possessed from 0 to 13 genes (in *P. chlororaphis*) coding for phytobeneficial functions, which were evenly distributed, regardless of the soil of origin (Table 26).

5.7.8. Additional genomic analyses of *Pseudomonas* strains

In this part of the study, putative BGCs found in the genomes of 29 *Pseudomonas* were further analyzed and manually curated. The highest number of putative BGCs was found in *P. chlororaphis* strains, up to 16 (from soil MI5; Table 27). The highest number of completed BGCs was three (in *P. chlororaphis* from soils MI3 and MI5 (both suppressive soils)).

Table 27. Number of putative biosynthetic gene clusters (BGCs) and number of BGCs with completion 1 or 1*, in *Pseudomonas* strains, found using the antiSMASH (Blin et al., 2019) within the MicroScope platform.

Soil	Species name from TYGS	Bacterial isolate	Number of putative BGCs	Number of BGCs with completion 1 or 1*
MI2	*P. siliginis*	IT-1P	8	0
	Pseudomonas GN-1	IT-2P	11	1
	Pseudomonas GN-2	IT-4P	8	0
	Pseudomonas GN-3	IT-12P	10	1
	P. jessenii	IT-43P	8	0
	Pseudomonas GN-4	IT-44P	8	0
	Pseudomonas GN-5	IT-74P	10	0
MI3	*Pseudomonas GN-6*	IT-100P	8	0
	Pseudomonas GN-6	IT-171P	9	0
	Pseudomonas GN-7	IT-176P	6	0
	P. serbica	IT-194P	12	0
	P. chlororaphis	IT-196P	15	3
	P. chlororaphis	IT-201P	14	3
MI4	*P. serboccidentalis*	IT-215P	9	0
	Pseudomonas GN-8	IT-218P	11	0
	P. brassicacearum	IT-228P	13	1
	Pseudomonas GN-9	IT-253P	7	1
	Pseudomonas GN-2	IT-P258	8	0
	Pseudomonas GN-10	IT-260P	11	1
	P. zeae	IT-265P	11	1
	Pseudomonas GN-11	IT-291P	9	0
	Pseudomonas GN-12	IT-294P	10	1
MI5	*P. chlororaphis*	IT-324P	16	2
	Pseudomonas GN-13	IT-347P	12	1
	P. marginalis	IT-357P	14	2

P. serbica	IT-P366	11	0
P. chlororaphis	IT-373P	15	3
P. serboccidentalis	IT-P374	11	0
Pseudomonas GN-14	IT-395P	11	1

* When two or more genes in a single MIBiG (The Minimum Information about a Biosynthetic Gene cluster database) curated region were similar, the same gene in MicroScope database can hit on these MIBiG genes. When this happens, the completion can be higher than 1 (represented by 1*).

Manual curation of putative BGCs found in *Pseudomonas* genomes using the antiSMASH revealed an operon controlling type VI secretion system in all *P. chlororaphis* strains, and an operon controlling type III secretion system in strain IT-324P (Table 28). In the *P. koreensis* subgroup, antiSMASH revealed an operon controlling type VI secretion system in both *P. serboccidentalis* strains. Strains *Pseudomonas GN-1* IT-2P (from MI2), *GN-10* IT-260P (from MI4), *GN-13* IT-347P (from MI5), *GN-14* IT-395P (from MI5), *P. zeae* IT-265P (from MI4) and *P. siliginis* IT-1P (from MI2) contained an operon controlling type VI secretion system, while *P. zeae* IT-265P also contained an operon controlling type III secretion system. In the *P. kielensis* subgroup, *Pseudomonas GN-7* IT-176P (from MI3) harbored an operon controlling type VI secretion system. In the *P. jessenii* subgroup, *P. serbica* IT-P366 (from MI5 soil) and IT-194P (from MI3 soil) host an operon controlling type VI secretion, and the megaplasmid of IT-P366 displays an operon controlling type IV secretion system. *Pseudomonas GN-2* strains IT-4P (from soil MI2) and IT-P258 (from soil MI4) contained an operon controlling type VI secretion system. Strains *GN-5* IT-74P, *GN-11* IT-291P and *GN-8* IT-218P also contained an operon involved in controlling type VI secretion system. In the *P. mandelii* subgroup, *Pseudomonas GN-6* IT-100P and IT-171P (both from soil MI3) displayed an operon controlling type III and type VI secretion systems. antiSMASH revealed a type VI secretion system – controlling operon in IT-44P, IT-294P and IT-253P, and a type III secretion system – controlling operon in IT-253P. In the *P. corrugata* subgroup, *P. brassicacearum* IT-228P (from soil MI4) had operons controlling type III and type VI secretion systems. Finally, in the *P. fluorescens* subgroup, *P. marginalis* IT-357P (from soil MI5) contained operons involved in controlling type III and type VI secretion systems.

Table 28. Putative biosynthetic gene clusters (BGCs) identified using antiSMASH (Blin et al., 2019) and manually curated. Dark green square shows the presence of BGC, pale green square shows the presence of BGC, but with different gene synteny, dark green triangle shows the partial presence of BGC and pale pink circle shows the absence of BGC. [a] BGC compared to the one present in IT-196P, [b] compared to IT-324P, [c] compared to IT-373P, [d] compared to IT-P258, [e] compared to IT-P4, [f] compared to IT-215P, [g] compared to IT-P374, [h] compared to IT-P366, [i] compared to IT-194P, [j] compared to *Pseudomonas ogarae* F113 and [k] as described in Costa et al. (2021). Annotation was completed on April 18th, 2023.

Bacterial isolate		Antimicrobial compounds							Siderophores					Metabolism/ cellular processes			Mot ility	Secretion systems		Mixed regions			Virulence/ pathogenesis		
		Massetolide A (cyclic lipopeptide) [a]	Pyrrolnitrin [a]	Phenazine [a]	Visconsin-like peptide synthesis [d]	Fragine-like peptide synthesis [a]	Putative bacitracin/enterobactin synthesis [a]	Achromobactin-like siderophore synthesis [a]	Pyoverdine-like siderophore synthesis 1 [a*]	Pyoverdine-like siderophore synthesis 2 [a*]	Rhizobactin-like siderophore biosynthesis [d]	IucA/IucC family siderophore biosynthesis [f]	Aryl polyene [a]	Cellular processes, signalling, signal transduction [e]	Cell wall synthesis/lipid metabolism [b]	Biofilm formation/chemotaxis [i]	Type III secretion system [j]	Type IV secretion system (Dot/Icm family) [k]	Type VI secretion system [j]	Pyoverdine synthesis/catabolism of citronellol and geraniol [c]	Lipoteichoic acid synthesis; putative cyanophycin synthesis-like [a]	Genes for lipid biosynthesis, signaling molecules and penicilin amidase [g]	Steroid degradation [h]	Potentially involved in bacterial pathogenesis: membrane sulfatase and ubiquitin transferase [h]
Reference strains																								
P. ogarae	F113	●	●	●	●	▢	●	●	▢	●	●	●	▢	●	●	◢	■	●	■	◢	■	●	●	●
P. protegens	CHA0	●	●	●	●	●	●	●	▢	●	●	●	■	●	●	◢	●	●	■	◢	■	●	●	●
MI2 soil																								
P. siliginis	IT-1P	●	●	●	●	▢	●	●	▢	●	●	●	■	▢	●	◢	●	●	■	◢	▢	●	●	●	■
Pseudomonas GN-1	IT-2P	◢	●	●	●	▢	●	●	▢	●	◢	■	■	■	●	◢	●	●	■	◢	■	●	●	●	■
Pseudomonas GN-2	IT-4P	●	●	●	■	▢	●	●	▢	●	■	●	▢	■	●	◢	●	●	■	◢	▢	●	●	●	●

Pseudomonas GN-3	IT-12P	
P. jessenii	IT-43P	
Pseudomonas GN-4	IT-44P	
Pseudomonas GN-5	IT-74P	
MI3 soil		
Pseudomonas GN-6	IT-100P	
Pseudomonas GN-6	IT-171P	
Pseudomonas GN-7	IT-176P	
P. serbica	IT-194P	
P. chlororaphis	IT-196P	
P. chlororaphis	IT-201P	
MI4 soil		
P. serboccidentalis	IT-215P	
Pseudomonas GN-8	IT-218P	
P. brassicacearum	IT-228P	
Pseudomonas GN-9	IT-253P	
Pseudomonas GN-2	IT-P258	
Pseudomonas GN-10	IT-260P	

P. zeae	IT-265P	
Pseudomonas GN-11	IT-291P	
Pseudomonas GN-12	IT-294P	
MI5 soil		
P. chlororaphis	IT-324P	
Pseudomonas GN-13	IT-347P	
P. marginalis	IT-357P	
P. serbica	IT-P366	
P. serbica plasmid	IT-P366	
P. chlororaphis	IT-373P	
P. serboccidentalis	IT-P374	
Pseudomonas GN-14	IT-395P	

* Pyoverdine-like siderophore synthesis region 1 is present in *P. chlororaphis* IT-196P from position 4223142 to 4276158 and pyoverdine-like synthesis region 2 from position 6268542 to 6339315.

5.7.9. *In vitro* biocontrol and plant growth promoting activity of *Pseudomonas* and correspondence between gene presence and *in vitro* activities

After the genome annotation, 29 *Pseudomonas* were tested *in vitro* for traits contributing to biocontrol or plant growth promotion. This included the assessment of *in vitro* siderophore production, production of HCN, production of lytic enzymes (proteases, chitinases and cellulases), phytohormones production, ACC deaminase production and solubilization of phosphates (Figure 33).

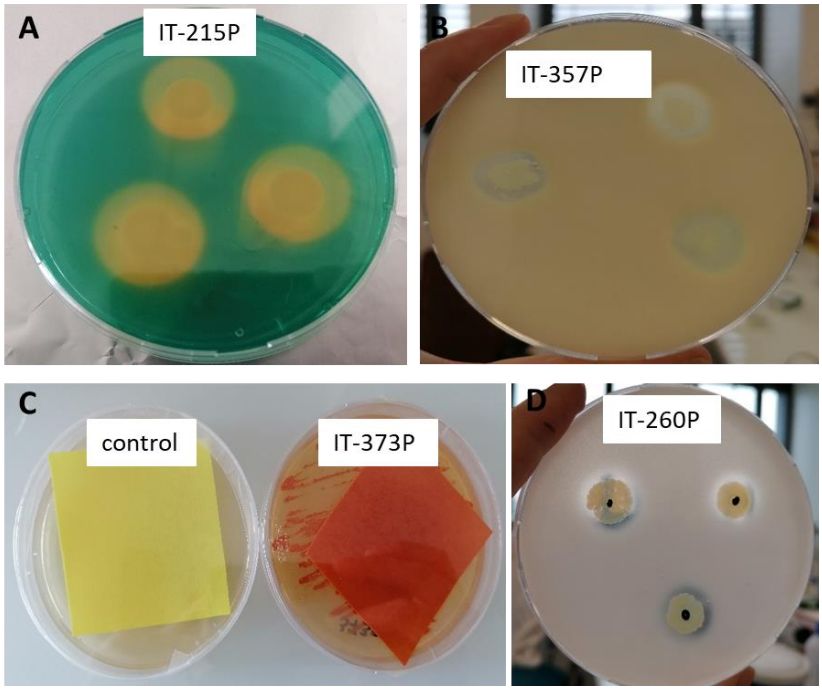

Figure 33. *In vitro* characterization of *Pseudomonas* strains. (A) *Pseudomonas serboccidentalis* IT-215P producing siderophores. (B) *Pseudomonas marginalis* IT-357P producing proteases. (C) Right: *Pseudomonas chlororaphis* IT-373P producing HCN; Left: Negative control. (D) *Pseudomonas GN-10* IT-260P with phosphates solubilizing activity.

When observing the distribution of exhibited biocontrol and plant-growth promoting properties within each *P. fluorescens* subgroup, in the *P. chlororaphis* subgroup, the MI3 isolates IT-196P and IT-201P and MI5 isolates IT-324P and IT-373P had the ability to produce HCN, siderophore, chitinase (except IT-324P), proteases (except IT-196P), but not cellulases (Table 29). They also produced IAA and indole-3-carboxylic acid (except IT-324P), trans-zeatin, isopentenyl adenosine and kynurenic acid, whereas trans-zeatin riboside and 6-benzylaminopurine were produced only by the strain IT-201P from soil MI3, and none of the strains from the *P. chlororaphis* subgroup solubilized phosphates. In the *P. koreensis* subgroup, all eight strains produced HCN, siderophores, chitinase, IAA and indole-3-lactic acid. All but *P. serboccidentalis* IT-215P (soil MI4) and *Pseudomonas GN-13* IT-347P (soil MI5) solubilized inorganic sources of P and produced indole-3-propionic acid. Proteases were produced by five strains (IT-1P from soil MI2, IT-215P and IT-265P from soil MI4, IT-347P and IT-395P from soil MI5), while cellulase was produced only by *P. siliginis* IT-1P (from soil MI2). Indole-3-carboxylic acid was produced by four strains (IT-215P and IT-260P from soil MI4, IT-P374 and IT-395P

from soil MI5), trans-zeatin by five strains (IT-1P and IT-2P from soil MI2, IT-215P, IT-260P and IT-265P from soil MI4, and IT-P374 from soil MI5), trans-zeatin riboside by three strains (IT-1P and IT-2P from soil MI2, IT-265P from soil MI4), 6-benzylaminopurine by four strains (IT-1P from soil MI2, IT-215P, IT-260P and IT-265P from soil MI4), isopentenyl adenosine by three strains (IT-1P and IT-2P from soil MI2, IT-215P from soil MI4), and kynurenic acid only by two strains (IT-2P from soil MI2 and IT-215P from soil MI4). In the *P. kielensis* subgroup, *Pseudomonas GN-7* IT-176P (soil MI3) was able to produce siderophores and proteases. It solubilized phosphates and produced the phytohormones trans-zeatin, trans-zeatin riboside, 6-benzylaminopurine, isopentenyl adenosine, abscisic acid and kynurenic acid. In the *P. jessenii* subgroup, *P. serbica* IT-P366 (from MI5) and IT-194P (from MI3) were able to solubilize phosphates and to produce siderophores, chitinase, and phytohormones (trans-zeatin, 6-benzylaminopurine, isopentenyl adenosine, abscisic acid and kynurenic acid), while only strain IT-P366 could produce trans-zeatin riboside. *Pseudomonas GN-2* IT-4P (from MI2) and IT-P258 (from MI4), displayed P solubilization, production of siderophores, chitinase, IAA, indole-3-lactic acid and indole-3-propionic acid, but only IT-P258 produced indole-3-carboxylic acid and IT-4P trans-zeatin. *Pseudomonas* sp. *GN-5* IT-74P (soil MI2), *GN-11* IT-291P (soil MI4) and *GN-8* IT-218P (soil MI4), and *P. jessenii* IT-43P exhibited production of siderophores, chitinase and trans-zeatin, and P solubilization. IAA, indole-3-lactic acid and indole-3-propionic acid were produced by all strains except IT-218P. Contrarily, indole-3-carboxylic acid, trans-zeatin riboside and kynurenic acid were produced only by IT-218P and proteases by IT-74P. In the *P. mandelii* subgroup, *Pseudomonas* sp. IT-100P and IT-171P (*GN-6*, both from soil MI3) produced siderophores, ACC deaminase, proteases, IAA, indole-3-lactic acid and indole-3-propionic acid, and they solubilized phosphate. Strain IT-171P also produced chitinase and indole-3-carboxylic acid. *Pseudomonas GN-3* IT-12P (soil MI2), *GN-9* IT-253P (soil MI4), *GN-12* IT-294P (soil MI4) and *GN-4* IT-44P (soil MI2) had in common production of chitinase, indole-3-propionic acid, trans-zeatin and trans-zeatin riboside, as well as phosphate solubilization. Siderophores were produced by all strains (except IT-294P), and IAA and indole-3-lactic acid by all strains (but IT-253P). Additionally, strains IT-12P and IT-44P produced ACC deaminase, 6-benzylaminopurine and isopentenyl adenosine. Proteases were produced only by IT-44P and indole-3-carboxylic acid only by IT-12P. In the *P. corrugata* subgroup, *P. brassicacearum* IT-228P produced HCN, siderophores, ACC deaminase, proteases, and several phytohormones. They included IAA, indole-3-lactic acid, indole-3-propionic acid, trans-zeatin, trans-zeatin riboside, 6-benzylaminopurine and isopentenyl adenosine. In the *P. fluorescens* subgroup, strain *P. marginalis* IT-357P solubilized phosphate and produced siderophores, ACC deaminase, proteases, cellulase, chitinase. It also produced the phytohormones indole-3-lactic acid, trans-zeatin, trans-zeatin riboside, 6-benzylaminopurine, isopentenyl adenosine and kynurenic acid.

Table 29. Correspondence between gene presence and *in vitro* activities involved in plant-growth promotion and biocontrol in 29 *Pseudomonas,* according to the soil of origin. Activity is marked with a green colour. Gene corresponding to a given activity *in vitro* (when found in the genomes) is indicated. Cellulase and chitinase were predicted using dbCAN2 (v.3; Zhang et al., 2018) and compared with the CAZy database using HMMER (v.3.3; Eddy, 2011). Prediction of function and substrate specificity of CAZyme families or subfamilies was performed based on a review of activities assigned to CAZymes with known structures (characterized enzymes) in the CAZy database (http://www.cazy.org) (Lombard et al., 2014) and manually curated, as previously described (López-Mondéjar et al., 2022).

Soil	Bacterial isolate	HCN	Siderophores	Phosphate solubilization	ACC deaminase	Protease	Cellulase	Chitinase	Indole-3-acetic acid	Indole-3-lactic acid	Indole-3-carboxylic acid	Indole-3-propionic acid	Trans-zeatin	Trans-zeatin riboside	6-Benzylaminopurine	Isopentenyl adenosine	Abscisic acid	Kynurenic acid	Number of phytobenefical functions
MI2	*P. siliginis* IT-1P	+		*gcd, gad*		+		+											13
	Pseudomonas GN-1 IT-2P	+	+	*gcd, gad*		+		+											10
	Pseudomonas GN-2 IT-4P		+	*gcd*				+	*iaaMH**										7
	Pseudomonas GN-3 IT-12P				+			+	*iaaMH**										12
	P. jessenii IT-43P		+	*gcd*				+	*iaaMH**										7
	Pseudomonas GN-4 IT-44P		+		+	+		+	*iaaMH**										12
	Pseudomonas GN-5 IT-74P		+	*gcd*		+		+											8
MI3	*Pseudomonas GN-6* IT-100P		+		+	+		+											7
	Pseudomonas GN-6 IT-171P		+		+	+		+											9
	Pseudomonas GN-7 IT-176P			*gcd*				+											9
	P. serbica IT-194P		+					+	*iaaMH**										8
	P. chlororaphis IT-196P	+	+	*gcd, gad*		+		+	*iaaMH*										8
	P. chlororaphis IT-201P	+	+	*gcd, gad*		+		+	*iaaMH*										11

MI4	*P. serboccidentalis* IT-215P	+	+	*gcd, gad*				+											12
	Pseudomonas GN-8 IT-218P		+	*gad*				+											7
	P. brassicacearum IT-228P	+			+	+		+	*iaaMH**										11
	Pseudomonas GN-9 IT-253P							+											6
	Pseudomonas GN-2 IT-P258			*gcd*				+											7
	Pseudomonas GN-10 IT-260P	+	+	*gcd, gad*				+											10
	P. zeae IT-265P	+	+	*gcd, gad*				+											11
	Pseudomonas GN-11 IT-291P			*gcd*				+											7
	Pseudomonas GN-12 IT-294P			*gad*		+		+											7
MI5	*P. chlororaphis* IT-324P	+	+	*gcd, gad*		+		+	*iaaMH*										5
	Pseudomonas GN-13 IT-347P	+	+	*gcd, gad*				+											7
	P. marginalis IT-357P			*gad*	+	+		+											12
	P. serbica IT-P366							+	*iaaMH**										9
	P. chlororaphis IT-373P	+	+	*gcd, gad*		+		+	*iaaMH*										9
	P. serboccidentalis IT-P374	+	+	*gcd, gad*				+											8
	Pseudomonas GN-14 IT-395P	+	+	*gcd, gad*				+											9

* *iaaH* found with only 30-40 % identity; none of the isolates produced indole-3-pyruvic acid, indole-3-butyric acid, tryptophol, kinetin, gibberellin A1 or gibberellic acid

Out of the activities tested *in vitro*, production of HCN and ACC-deaminase activity by *Pseudomonas* strains from all four soils paralleled well with the presence of the corresponding genes (Table 29). All but *Pseudomonas GN-12* IT-294P from MI4 and *P. chlororaphis* IT-324P from MI5 produced siderophores *in vitro*. *In vitro* phosphate solubilization activity corresponded to the presence of *gcd* and/or *gad* genes in 15 of 22 strains, but not in seven other strains, indicating other P solubilization mechanisms. In addition, six strains possessed both *gcd* and *gad*, but did not solubilize phosphate under the conditions tested. Similarly, the presence of *aprA* matched with the *in vitro* proteolytic activities in 10 strains, while in five strains activity was present but not *aprA*, suggesting the involvement of other protease genes. In three strains, *aprA* gene was found, but without activity. Most strains produced chitinases, in accordance with the presence of chitinase genes (Figure 32). Cellulase activity was found only in *P. siliginis* IT-1P (which displays GH3 family genes acting on cellobiose) and *P. marginalis* IT-357P (with genes encoding cellulose degradation) (Figure 31 and Figure 32). Most strains produced IAA, but only eleven of them harbored *iaaMH* genes coding for auxin synthesis, and none of the isolates harbored *ipdC* or *ppdC* genes. Indole-3-pyruvic acid, indole-3-butyric acid, tryptophol, kinetin, gibberellin A1 or gibberellic acid was not produced by any of the strains, while all the other phytohormones tested were produced by strains from all soils.

In conclusion, all phenotypic traits tested were found in isolates from all four soils, and strains with higher number of phytobeneficial functions (11, 12 or 13) were isolated from all four soils. Correspondence between gene presence and *in vitro* activity matched in the case of HCN and ACC-deaminase productions, where all the gene(s) involved in the pathway are known. However, for production of siderophores, proteases, cellulases and chitinases, P solubilization, and IAA production, gene presence did not coincide with the activity in all strains, indicating either the involvement of other genes in these biochemical functions (in cases when an activity was reported, but the gene(s) searched for were not found) or lack of gene expression (in cases when the gene was present, but the activity could not be detected).

5.7.10. Inhibitory effect of *Pseudomonas* volatile organic compounds (VOCs) on growth and inhibitory effect of *Pseudomonas* exudates on sporulation of *Fusarium graminearum* Fg1

In this part of the study, the inhibitory effect of VOCs produced by *Pseudomonas* strains towards *F. graminearum* Fg1, as well as the ability of *Pseudomonas* exudates to inhibit sporulation of *F. graminearum* Fg1 in liquid medium, were tested. Growth inhibition of *F. graminearum* Fg1 by VOCs produced by *Pseudomonas* strains was >20% with *P. marginalis* IT-357P (31.6%) (Figure 34) and *Pseudomonas GN-14* IT-395P (30.6%) from soil MI5 and *P. serboccidentalis* IT-215P (21.5%) from soil MI4. Inhibition was below 20% for *P. brassicacearum* IT-228P (15.4%) and *Pseudomonas GN-9* IT-253P (14.0%) from soil MI4, and *Pseudomonas GN-1* IT-2P (14%) and *P. chlororaphis* IT-196P (18.9%) from soils MI2 and MI3.

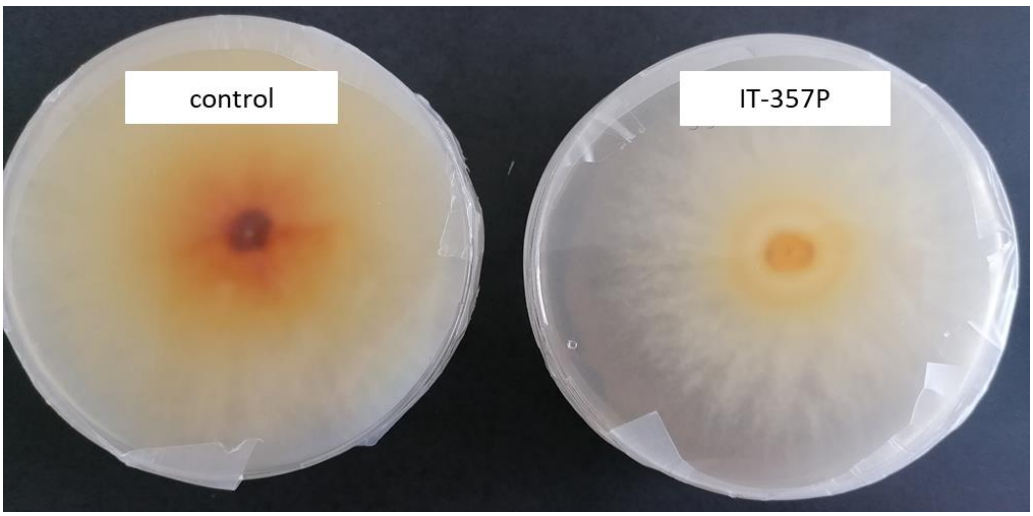

Figure 34. Ability of *Pseudomonas marginalis* IT-357P to inhibit mycelial growth of *Fusarium graminearum* Fg1 through production of VOCs. Left: Negative control, i.e., *Fusarium graminearum* Fg1 only; Right: *Pseudomonas marginalis* IT-357P inhibiting growth of *Fusarium graminearum* Fg1.

Additionally, a microplate assay was used to test the ability of *Pseudomonas* exudates to inhibit conidia germination of *F. graminearum* Fg1. This kind of spore germination inhibition was only observed with the two strains from MI5 soil (13.5% with *P. marginalis* IT-357P and 18.1% with *Pseudomonas GN-14* IT-395P).

In summary, from the 29 *Pseudomonas* tested, seven of them (originating from soils MI2, MI3, MI4 and MI5) were able to inhibit growth of *F. graminearum* Fg1 via production of VOCs, and two of them (both from MI5 soil) were able to inhibit fungal spore germination.

5.7.11. *In planta* effects of *Pseudomonas* on wheat inoculated with *Fusarium graminearum* Fg1

Pseudomonas strains chosen for *in planta* assay included strains belonging to *P. chlororaphis* and *P. brassicacearum* species, since these are known for their phytobeneficial properties, i.e., *P. chlororaphis* IT-196P and *P. chlororaphis* IT-201P from soil MI3, *P. brassicacearum* IT-228P from soil MI4, and *P. chlororaphis* IT-324P and *P. chlororaphis* IT-373P, from soil MI5 (Figure 35). In the *in planta* assay performed with the soil LCSA, the addition of *F. graminearum* Fg1 alone resulted in a significantly lower number of germinated seeds at 14 days (Figure 35A), high disease symptoms (Figure 35B), lower biomass (Figure 35C) and lower chlorophyll rate at 45 days (Figure 35D), in comparison with non-inoculated seeds. In comparison with seeds inoculated with *F. graminearum* Fg1, there was a trend towards a higher number of germinated seeds when inoculation was carried out with *P. chlororaphis* IT-373P (from soil MI5) and *P. brassicacearum* IT-228P (from soil MI4), but this trend was not significant at $P < 0.05$. In addition, bacterial inoculation resulted in high disease symptoms with all the *Pseudomonas* tested, similar to seeds inoculated with *F. graminearum* Fg1. Finally, biomass and chlorophyll rate of germinated plants were always the same or lower, compared to the seeds inoculated with *F. graminearum* Fg1.

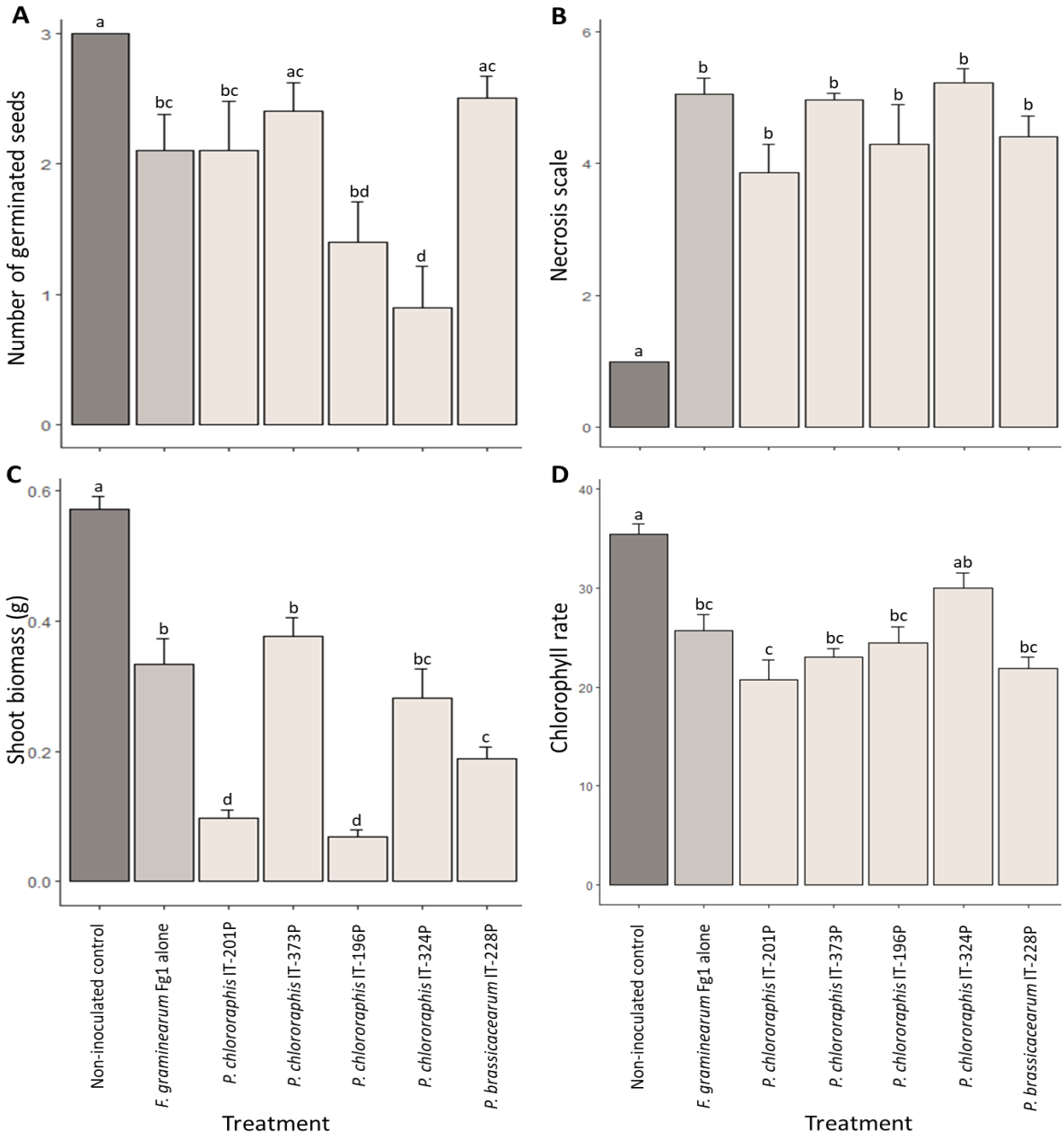

Figure 35. Results of the *in planta* protection assay. (A) Number of germinated seeds at two weeks after inoculation with *Pseudomonas* and *Fusarium graminearum* Fg1. Results are presented as mean + standard error. Data were analyzed using Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with letters a to d. (B) Disease symptoms of crown-rot at 45 days after inoculation with *Pseudomonas* and *F. graminearum* Fg1. Non-germinated plants were regarded as missing data (NA). Results are presented as mean + standard error. Data were analyzed using Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with significance letters a and b. (C) Shoot biomass of wheat plants at 45 days after inoculation with *Pseudomonas* and *Fusarium graminearum* Fg1. Non-germinated plants were regarded as missing data (NA). Results are presented as mean + standard error. Data were analyzed using Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with letters a to d. (D) Chlorophyll rate of wheat plants at 45 days after inoculation with *Pseudomonas* and *Fusarium graminearum* Fg1. The chlorophyll rate of each wheat plant was the average of three measurements, taken on the 5th, 6th and 7th grown leaf. Non-germinated plants and plants without grown leaves were regarded as missing data (NA).

Results are presented as mean + standard error. Data were analyzed using ANOVA and Tukey's test ($P < 0.05$), and statistical differences are shown with letters a to c.

In summary, *P. chlororaphis* IT-373P (from suppressive soil MI5) and *P. brassicacearum* IT-228P (from non-suppressive soil MI4) enhanced wheat germination (although this was not statistically significant), but did not protect wheat plant from crown rot disease, nor could prevent the decrease in shoot biomass and chlorophyll rate. All other *Pseudomonas* tested in *in planta* assay did not contribute to wheat phytoprotection.

6. DISCUSSION

Cereal grains (wheat, corn, rice, barley, sorghum, oats and rye) are the most produced crops worldwide, substantially supplying energy to humans and livestock (Fatima et al., 2020). For example, in 2018, wheat provided 20% of all the calories consumed by humans ("Food and Agriculture Organization of the United Nations", 2020). In the last decade, with growing human population and demand for cereal grains, agricultural management has become increasingly intensified, with excessive use of fertilizers and pesticides, leading to declined soil microbial diversity or changes in microbial community structure (Gupta et al., 2022). These factors, together with the ongoing climate change, have paved a way for even higher intrusion of pests and diseases, which can spread quickly and cause significant yield losses (Ramankutty et al., 2018). Wheat, for example, experiences up to 23% yield loss due to pests and pathogens worldwide (Savary et al., 2019), with *F. graminearum* as one of the predominant causal agents (Yli-Mattila, 2010; Nielsen et al., 2012; West et al., 2012). In the context of global climate warming, the relationship between biodiversity and crop health has received more attention with the emergence of different pathogens/pests (Trębicki et al., 2017). By exploring the existing mechanisms underlying soil suppressiveness, and trying to learn from Nature, the humanity has been given an opportunity to invent nature-based strategies to control fungal pathogens.

6.1. Fungistasis and soil suppressiveness to *Fusarium graminearum* diseases

Soils naturally suppressive to soil-borne pathogens have been recognized worldwide more than 70 years ago (Vasudeva and Roy, 1950; Alabouvette, 1986; Schlatter et al., 2017; Lv et al., 2023). However, in many countries of the world and for the majority of soil-borne pathogens, the distribution of disease suppressiveness is undetermined because of the absence of simple tools that will enable reliable identification of such soils. Fungistasis is a significant component of soil suppressiveness as it contributes to decrease in the amount of fungal inoculum available for disease development in plants (Garbeva et al., 2011). Therefore, fungistasis is an important trait, but it remains insufficiently understood. At the beginning of this research, 26 manured and non-manured soils were sampled from different regions in Serbia, i.e., from regions in northern, plain part of Serbia, where the agriculture is more intensive because of the existence of fertile soil of type chernozem, and in western and central, hilly parts of Serbia, where the agriculture is more traditional (Tanasijević et al., 1964; Nejgebauer et al., 1971). Firstly, these 26 soils were screened for fungistasis to *F. graminearum*. Fungistasis potential was found in 10 of the screened soils (38%), where the amount of fungal DNA declined, and all of these soils were from western/central Serbia, where the agricultural practices are more compatible with biodiversity protection. This decline was due to antifungal properties of the soil microbiota, as *F. graminearum* grew readily when these 10 soils were sterilized, to the same extent as in the non-fungistatic soils, highlighting the microbial basis of soil fungistasis. The infectious cycle of *F. graminearum* includes a phase where the pathogen must survive in the soil before infecting new seedlings (Pereyra et al., 2004; Cobo Díaz et al., 2019), and all natural soils can exert some level of pathogen control due to the presence of an active microbiota (Lockwood, 1977), although without necessarily achieving fungistasis. Contrarily, none of the fungistatic soils were found in northern parts of Serbia (soils SO and NK), where soils are of type chernozem and agriculture is more intensive. This could be due to long-term agricultural exploitation of these soils (despite using manure amendments at some fields), that led to deterioration of biological soil properties (Gupta et al., 2022) and, consequential, higher infestation after inoculating *F. graminearum.* Besides farming practices that can affect the survival of phytopathogens in soil (Legrand et al., 2017; Supronienė et al., 2023), microbial communities may vary with different

soil types (Karimi et al., 2020). Out of 10 fungistatic soils of interest, seven of them (70%) had been amended with manure, and manure treatments have been shown as a significant factor promoting fungistasis, especially in soils from Mionica (MI), with a similar trend in soils near Čačak (CA). This is in line with research by Legrand et al. (2019), that showed a positive relation between manure amendments and fungistasis potential. Animal manure amendments bring new microorganisms, supply nutrients to the soils and impact the resident microbiota (Mousa and Raizada, 2016). However, this was still not sufficient to develop fungistasis in the chernozems of northern Serbia (soils SO and NK), suggesting that, in this case, manure amendments could not restore deteriorated soil biodiversity and promote fungistasis. At locations near Novi Karlovci (NK), there was even a higher amount of fungal DNA in manured than in non-manured soils, demonstrating that manure amendments may also negatively affect pathogen suppression, as shown in a study by Termorshuizen et al. (2006), although this is a rare occurrence. Moreover, it is possible that sheep manure, used only in soils near Mionica, better promotes fungistasis than beef and chicken manure, used at other locations, as it is known that type of manure amendment largely affects its efficiency (Janvier et al., 2007). In soils near Valjevo (VA), there was a decrease of fungal DNA in both manured and non-manured soils, possibly explained by the fact that indigenous microbiota of certain VA soils is enough to provide pathogen suppression and to resist to ecosystem perturbations provoked by the exogenous addition of fungal pathogen and manure amendments.

Furthermore, using soils from Mionica, the potential of fungistatic soils to also be suppressive to *F. graminearum* damping-off disease of wheat was tested. This possibility has been mentioned in earlier works (Lockwood, 1977; Garbeva et al., 2011), but to our knowledge, this was the first study that had a two-fold approach and performed both fungistasis and *in planta* suppressiveness assays. Fungistasis and suppressiveness assay results matched for three out of four MI soils, i.e., soils MI2 and MI3 (manured) were both fungistatic and suppressive, soil MI4 (non-manured) was non-fungistatic and non-suppressive, whereas soil MI5 (non-manured) was non-fungistatic, but suppressive. In the case of manured MI2 soil (fungistatic and suppressive), it was observed that plants' dry shoot biomass and shoot density were significantly lower in *F. graminearum Fg1*-inoculated than in non-inoculated soils. Although this soil was fungistatic towards *F. graminearum* Fg1 and had the ability to suppress pathogen growth, it is possible that due to the presence of some other soil-borne pathogen(s) in the soil, that were attracted by the plant's exudates, the plants' biomass and density decreased, as all of the defense mechanisms were oriented towards phytoprotection from the high-pressure inoculum of *F. graminearum* Fg1. It is widely known that soil suppressiveness to one pathogen sometimes means higher susceptibility to the other(s) (Schlatter et al., 2017). In the case of other manured soil, MI3, there was no difference in plants' biomass, shoot length or density between *F. graminearum* Fg1-inoculated and non-inoculated soils, suggesting that phytoprotective capacity of this soil was not at the expense of plant fitness. An example of a non-manured soil, MI4, that was non-fungistatic and non-suppressive, demonstrated a complete lack of soil's ability to suppress the pathogen and subsequent development of plant infection. Finally, the case of MI5 soil may be explained in several ways. Firstly, it can be a result of ISR-triggering rhizosphere microbiota, as previously described in tobacco exposed to the black root rot pathogen *T. basicola* (Almario et al., 2014), or in carnation (Van Peer et al., 1991), tomato (Tamietti et al., 1993) or radish (Leeman et al., 1995), confronted to the Fusarium wilt pathogen *F. oxysporum*. Secondly, it can be a result of direct pathogen inhibition by the rhizosphere microbiota (including *Pseudomonas*) on roots, where certain antagonistic microbial populations may be enriched due to roots exudation (Kyselková and Moënne-Loccoz, 2012). Thirdly, it can be a result of an action of root-inhabiting PGPR that help plant nutrient acquisition, thus leading to an improved fitness and increased tolerance to abiotic stresses (Glick, 2012). The latter was confirmed with wheat shoot biomass, length and density being overall higher in manure-amended soils, than in non-

amended soils, as found before (Ibrahim et al., 2008). This is also in line with previous research that highlighted the significance of organic and compost amendments in enhancing soil phytoprotection capacity against soil-borne pathogens (Mousa and Raizada, 2016; Mitsuboshi et al., 2018; Nguyen et al., 2018). Although at all fields, wheat was grown in a crop rotation, at the time of sampling MI soils for fungistasis assay, fields were grown with alfalfa (MI2), sunflower (MI3), meadow (MI5) and wheat (MI4), while at the time of the sampling MI soils for suppressiveness *in planta* assay, fields were grown with maize (MI2), wheat (MI3, MI5) or were left as a meadow (MI4). In the case of MI5 soils, as wheat was grown at the time of soil sampling (spring 2021) for suppressiveness assay and ITS and 16S rRNA metabarcoding analysis, it is possible that this contributed to the enrichment of microbial populations positively impacting the wheat plant, inducing ISR in plant, and/or suppressing *Fusarium*, compared to fungistasis assay, where at the time of soil sampling (autumn 2020) there was a meadow. These differences between the two soil samplings (different timing and different crops present in the fields) likely contributed to the differences in microbiota. Previous studies already outlined the impact of crops and time of sampling on soil microbiota, and suggested changes in microbiota composition and activity (Sánchez-Cañizares et al., 2017; Luo et al., 2020).

Fungistasis and suppressiveness assays enabled distinguishing three soil categories and based on these results, one soil from each of the three categories was chosen, i.e., soil MI4 (non-fungistatic and non-suppressive), soil MI5 (non-fungistatic and suppressive) and soil MI2 (fungistatic and suppressive). In order to uncover fungal and prokaryotic populations responsible for such a distinction, ITS and 16S rRNA-based taxonomic profiling were performed. A wide range of bacterial and fungal taxa may be involved in disease suppression (Kloepper et al., 1980; Tamietti and Alabouvette, 1986; Weller et al., 2002; Ossowicki et al., 2020; Yadav et al., 2021). However, here the results demonstrated a lack of a specific fungal or prokaryotic group that is enriched only in suppressive soils, rather that microbiota was soil category-specific, with shared core microbiome. Indeed, the most abundant prokaryotic and fungal phyla found in all three soil categories were the same, as already suggested by Simonin et al. (2020), who highlighted that many microbial taxa are continuously associated to the wheat rhizospheres across different soils. Taxon from the genus *Rubrobacter,* found only in suppressive soil MI2, was also documented in significantly higher abundance in soils suppressive to Fusarium wilt from Châteaurenard (Siegel-Hertz et al., 2018), however it was not documented in an another suppressive soil (MI5), used in this study. This could be due to the fact that soil MI2 was manure-amended, while MI5 was not, and, as already mentioned before, manure serves as a source of new microbial mass (Mousa and Raizada, 2016). Another specificity of manured soil MI2 was that, upon inoculation with *F. graminearum* Fg1, species evenness was higher in this soil, compared to soils MI4 and MI5. Higher eveness contributes to better ecosystem resilience and improved resistance to pathogenic invasions (De Roy et al., 2013), which might have promoted the suppressive character of soil MI2. Genus *Sphingomonas* was only observed in conducive soil MI4, which is reminiscent of a taxon from the order *Sphingomonadales*, significantly enriched in soils conducive to banana wilt disease in Hainan, China (Shen et al., 2022). When it comes to fungal community, it was observed that taxa from the genus *Neocosmospora* and family *Didymellaceae* can be found in both suppressive soils MI5 and MI2, however, both of these contain sapropythic and pathogenic species, and their relation to soil suppressiveness remains unclear (Chen et al., 2017; Sandoval-Denis et al., 2019). Inoculation with *F. graminearum* Fg1 resulted in a lower fungal species evenness in soil MI4, evidencing that this ecosystem is more prone to disturbances (De Roy et al., 2013).

It is well known that plant microbiota is acquired from the surrounding soil environment and that plants recruit their microbiota with their exudates, that are determined by plant species and variety, as well as developmental stage (Sánchez-Cañizares et al., 2017).

Studies also suggested that microbiota can be shaped by the soil properties (i.e., availability of nutrients and carbon, pH; Custódio et al., 2022) and evolutionary history of plants (Bouffaud et al., 2016; Simonin et al., 2020). Furthermore, microbes and microbial communities are constantly evolving and adapting to dynamically changing ecological and biotic conditions, in order to survive, the latter being defined as the "Red Queen hypothesis" (Van Valen, 1977). As shown during this research, when biotic conditions in rhizosphere soils were changed due to inoculation with *F. graminearum,* the rhizosphere prokaryotic and fungal community also changed. More specifically, upon *F. graminearum* inoculation, relative rhizosphere abundance of taxa from the phyla *Chytridiomycota* increased in soil MI4, *Firmicutes* increased in soils MI4 and MI5, while in soil MI2, fungal inoculation resulted in an increased relative abundance of *Actinobacteriota* and *Proteobacteria*, but led to lower levels of *Mortierellomycota*, *Crenarchaeota* and *Chloroflexi.* This was probably due to antagonistic interactions between the resident microbiota and the added pathogen and/or changes in plant metabolism and exudates (Rojas et al., 2014). This change in quantity and composition of plant root exudates due to pathogen inoculation is termed "cry for help" strategy, when plants recruit microbes with biocontrol properties (Rizaludin et al., 2021). For example, it was shown that inoculation of barley plants with *F. graminearum* triggered changes in root exudates, where roots started producing different antifungal organic acids (Lanoue et al., 2010), that also act as attractants for fluorescent pseudomonas, and in such a way, barley plants manipulated their rhizosphere microbial community composition (Oku et al., 2014). Similarly, inoculation of *Carex arenaria* plant with *F. culmorum*, provoked changes in composition of VOCs produced by plant roots, and attracted microbes with antifungal properties (Schulz-Bohm et al., 2018).

As expected, relative abundance of *F. graminearum* was significantly higher in inoculated than in non-inoculated soils. In soil MI2, *F. graminearum* was evidenced in the rhizosphere albeit at a lower relative abundance than in the other soils from Mionica. However, in the second suppressive soil, MI5, *F. graminearum* was among the most abundant representatives of the *Fusarium* genus, pointing to the importance of rhizosphere interactions for wheat protection in soil MI5. As already explained before, health of plants grown in *F. graminearum*–rich soil MI5 may be a result of rhizosphere microbiota triggering ISR in plants (Almario et al., 2014), direct inhibition of the fungal pathogen on plant roots (Kyselková and Moënne-Loccoz, 2012), and/or presence of rhizospheric PGPR that may increase plants' resistance towards fungal stress (Glick, 2012). It is important to note that *F. graminearum* was also observed in non-inoculated soils, contrary to the qPCR data obtained during the fungistasis assay, where *F. graminearum* was not observed in non-inoculated soils. There are several explanations for this. Firstly, as mentioned before, sampling seasons and crops were different between the sampling for fungistasis assay (autumn 2020) and sampling for suppressiveness assay and 16S rRNA gene and ITS metabarcoding (spring 2021), and this may have influenced the relative abundance of *Fusarium* (Bateman and Murray, 2001). Secondly, fungal metabarcoding was done using the ITS, a common barcode for identification of fungi, which is insufficiently informative for a subset of closely related *Fusarium* species. Phylogenetically, closely related *Fusarium* species, that diverged less than 10 million years ago, differ in only one or a few nucleotides in the ITS region, making it difficult to make a distinction between them, and contributing to somewhat of a bias when using this method of identification (O'Donnell et al., 2013; Hafez et al., 2020). Nevertheless, metabarcoding using the ITS is a simple method that enabled comparison of relative abundances of *Fusarium* species in different soils and conditions in this study. However, in order to be able to interpret the results more clearly, it is advised to sequence one (or more) of the following genes: translocation elongation factor-1α (*tef-1α*), RNA polymerase 1 and 2 (*rpb1* and *rpb2*), β-tubulin (*tub*), histone (*his*), ATP citrate lyase (*acl1*) or calmodulin (*CaM*) (Herron et al., 2015; Summerell, 2019; Crous et al., 2021).

In a dynamic system that consists of the holobiont (plant with its microbiome), phytopathogen and surrounding environmental factors, all three components determine the suppressive nature of soils, as defined by Jayaraman et al. (2021) through a disease triangle concept. Another issue is that in the natural ecosystems, there rarely exists a need to control a single pathogen, but, rather, there is an entire myriad of soil-borne pathogens. For example, there was an attempt to modify the soil microbiota with the *Brassica napus* seed meal amendments, aiming to induce suppressiveness towards apple replant disease caused by the phytopathogen *R. solani*. This approach successfully suppressed *R. solani*, but increased populations of *Pythium* spp. (Mazzola, 2007). Ideally, in order to profoundly understand soil suppressiveness to soil-borne diseases, future research should study all three factors from the triangle concept in parallel, aiming to better understand correlation between these factors and disease suppression. Due to the fact that this research offers insight into the soil fungistasis and suppressiveness to *F. graminearum* Fg1 wheat disease at the same time, data obtained in this study might aid in deciphering how the soil microbiome, that serves as a source of microbes for the plant microbiome, can be manipulated, aiming to achieve soil suppressiveness that is customized to the plant, pathogen(s) and the surrounding environmental factors.

6.2. Fungistatic soils as a source of bacteria with biocontrol properties against *Fusarium graminearum*

The rhizosphere represents a source of microorganisms that may control *Fusarium* (Wang et al., 2015; Jangir et al., 2018), especially if biocontrol strains are sought in disease-suppressive soils (Weller et al., 2007). In fungistatic soils, general soil suppressiveness is conferred via a range of competitive and other interactions between the soil microbiota and the pathogen (Garbeva et al., 2011; de Boer et al., 2019). Having this in mind, fungistatic soils have been neglected as a pool of plant-protecting microorganisms, including against *Fusarium* diseases (Stutz et al., 1986; Lemanceau and Alabouvette, 1991; Fuchs et al., 1997; Raaijmakers and Weller, 1998). To our knowledge, this was the first study that assessed the usefulness of both fungistatic and non-fungistatic soils as a source of biocontrol agents. Here, the isolates from both fungistatic and non-fungistatic soils were tested in a dual-culture assay with *F. graminearum* Fg1. A dual-culture assay is a common screening procedure when looking for potential biocontrol agents (Paulitz et al., 1992; Besset-Manzoni et al., 2019) and, here, it yielded 23 antagonistic bacteria, enabling the conclusion that both fungistatic and non-fungistatic soils are a good source of potential biocontrol agents. Surprisingly, none of the isolates from the phylum *Actinomycetota* (formerly *Actinomycetes*) could inhibit *F. graminearum* Fg1 on plates, despite the fact that this phylum is known for production of antibiotics (Cuesta et al., 2012) and lytic enzymes (Soltanzadeh et al., 2016). However, most of the *Actinomycetota* with antifungal properties are acidophils, while isolation media used in this study was targeting alkalophilic representatives (Poomthongdee et al., 2015), which could have contributed to the obtained results.

Whole genome sequencing is useful in clarifying the taxonomic status and general ecology of promising isolates (Zwolinski, 2007), as illustrated by the identification of as many as eight novel genomospecies from five genera (including well-studied *Pseudomonas*) among the 23 antagonistic strains. Whole genome sequencing of these 23 antagonistic bacteria showed that they belong to seven genera, i.e., *Pseudomonas* (10 strains), *Bacillus* (six strains), *Priestia* (formerly within *Bacillus*; two strains), *Brevibacillus* (one strain), *Burkholderia* (two strains), *Kosakonia* (one strain) and *Chryseobacterium* (one strain). As expected, representatives from the genera *Pseudomonas* and *Bacillus* were the most abundant in this collection, which is in line with previous screening studies (Janssen, 2006; Prashar et al., 2014; Nwachukwu et al., 2021). This was the case in both fungistatic and non-fungistatic soils,

indicating that there was not a major taxonomic bias in the procedure. Previous research on biocontrol agents against *Fusarium* rarely included *Brevibacillus* (Johnson et al., 2020), *Burkholderia* (Ho et al., 2015), *Chryseobacterium* (Khan et al., 2006) and *Kosakonia* (formerly *Enterobacter*) (Tsuda et al., 2001), however, their representatives were selected as potential biocontrol agents against *F. graminearum* and included in this research.

Whole genome sequencing and annotation is also a useful approach to probe the genetic potential of promising isolates, so this should become a standard procedure when analyzing potential biocontrol agents (Cai et al., 2017; Nelkner et al., 2019). Here, it was evidenced that *P. chlororaphis* strains IT-51CA3 and IT-162MI3 harbored genes that encode antifungal metabolites, such as phenazine, HPR, pyrrolnitrin and HCN, as did *P. chlororaphis* IT-48CA2, pointing that these properties are related to taxonomy, as previously documented in this species (Calderón et al., 2013; Loewen et al., 2014). The *in vitro* analysis of HCN production in *Pseudomonas*, which is a broadspectrum antimicrobial metabolite (Ramette et al., 2003), confirmed its activity in eight isolates, including the three *P. chlororaphis* strains. Only *P. brassicacearum* IT-43CA1 harbored genes for DAPG production, a key biocontrol property occurring in *P. brassicacearum* and other *Pseudomonas* species (Almario et al., 2017). It is also known that certain DAPG-producing *Pseudomonas* may elicit ISR in plants by producing this metabolite (Bakker et al., 2007). None of the *Priestia*, *Bacillus* and *Brevibacillus* isolates harbored *hcnABC* genes, nor showed their activity. Two *Burkholderia* isolates harbor genes for the production of pyrrolnitrin (as confirmed by antiSMASH), which is an antifungal antibiotic, as already described in a study by Deng et al. (2016). Besides the production of antifungal compounds, it is already known that a suppression of fungal pathogens can also occur through the siderophore production, which chelate iron, making it unavailable to the pathogen (Beneduzi et al., 2012). Analysis of siderophore biosynthesis revealed the production of siderophores in all the *Pseudomonas* isolates, including those that do not have the *pvdL* or *pchABCDEF* genes. However, this is explained by the fact that *Pseudomonas* species harbor a vast array of siderophores other than pyoverdine and pyochelin, such as pseudobactin, whose production is encoded by different genes that we did not search for (Mercado-Blanco et al., 2001; Leoni et al., 2002), and, due to their versatility, siderophores can be used as taxonomic and phylogenetic markers (Meyer, 2010). Lytic enzymes, such as proteases, cellulases and chitinases are also known to play role in antagonism towards *Fusarium* (Rathore et al., 2020). *In vitro* inspection of isolates showed that almost all of them produce proteases, even the isolates which do not contain the *aprA* gene which was searched for, coding for alkaline metalloproteinase production. This can be explained by the fact that bacteria produce proteases from two other families: serine and cysteine protease family (and rarely from aspartic protease family) (Sumantha et al., 2006; Zhou et al., 2009), which are all coded by different genes (Huyen et al., 2009; Mahmoud et al., 2021), and search for these genes should be included in future studies. Two *Priestia* isolates synthesize cellulase without having the cellulase genes, explainable by the presence of genes coding for β-glucosidases in their genomes, which are responsible for cellobiose (cellulose dimer) degradation (Zang et al., 2018). Chitinase activity was found in almost all the isolates from this study, as well as the genes for chitinase production, except in the case of *Pseudomonas GS-6* IT-196MI5, that possesses genes from the AA10 family, including lytic polysaccharide monooxygenases (LPMOs), potentially targeting chitin (Chaplin et al., 2016). Finally, gene *fitD*, coding for the insect toxin (*fluorescens* insecticidal toxin), is found in one isolate, *P. chlororaphis* IT-48CA2, which is in agreement with the presence of this gene in *P. chlororaphis* Pf-5, O6 and 30–84, as found in one study (Rangel et al., 2016).

Besides genes involved in biocontrol, some studied isolates possess gene clusters involved in modulation of plant hormonal status, such as those involved in ethylene and auxin biosynthesis, as well as ACC deaminase production. Testing the synthesis of IAA, showed that all *Pseudomonas* (except *Pseudomonas GS-5* IT-194MI4), and all *Bacillus* (except *B.*

licheniformis IT-74MI3), *Priestia* isolates, *Chryseobacterium* isolate and *Kosakonia* isolate have the ability of producing IAA, even without possessing *ipdC* or *iaaMH* genes. This is in accordance with the literature data, as it is known that there are more pathways for the IAA synthesis, than only through the indole-3-acetamide pathway, coded by the *ipdC* and *iaaMH* genes, however, not all of the genes and/or pathways are known to date (Patten et al., 2013). Besides IAA, bacteria from the collection were screened for production of other auxin phytohormones, cytokinins, gibberellins, abscisic acid and kynurenic acid. Majority of the *Pseudomonas* isolates were able to synthesize indole-3-propionic acid and trans-zeatin, while some of them were able to synthesize indole-3-lactic acid, indole-3-carboxylic acid, isopentenyl adenosine and kynurenic acid, which is not surprising for *Pseudomonas* species (Wurst et al., 1984; Akiyoshi et al., 1987; Pallai et al., 2012; Bortolotti et al., 2016). However, only *B. pseudomycoides* IT-40CA3 produced indole-3-pyruvic acid, a precursor of IAA (Patten et al., 2013) and none of the isolates produced indole-3-butyric acid, trans-zeatin riboside, kinetin, 6-benzylaminopurine, gibberellin A1, gibberellic acid and abscisic acid under the tested conditions. Tryptophol was produced only by *P. megaterium* IT-180MI3 and *K. quasisacchari* IT-91MI3, as documented in many other bacterial species (Palmieri and Petrini, 2019). Activity of the ACC deaminase, which is found in the isolate *P. brassicacearum* IT-43CA1, *Burkholderia GN-4* IT-111MI5 and *B. ambifaria* IT-158MI4, all three harboring the *acdS* gene, is responsible for cleaving the ACC, consequently lowering the level of ethylene in plants under the stress conditions (Saravanakumar and Samiyappan, 2007). Acetoin and 2,3-butanediol, products of mixed-acid fermentation and the plant-growth promoting compounds (Ryu et al., 2003), are often produced in *Pseudomonas*, as outlined by Loper et al. (2012). Acetoin and 2,3-butanediol synthesis genes, then genes responsible for 2,3-butanediol conversion to acetoin and genes involved in acetoin catabolism, were all found in the *Pseudomonas* isolates from this study. In the case of *Bacillota*, i.e., *Bacillus* and *Brevibacillus*, then *Kosakonia* and *Burkholderia* isolates, genes involved in acetoin and 2,3-butanediol metabolism were registered in the genome, as these are also frequent in these species (Li et al., 2017; Petrov and Petrova, 2021; Alvarez et al., 2022). Further genomic inspection of isolates found genes for inorganic phosphate solubilization in almost all *Pseudomonas* and all *Burkholderia* isolates, one more property already documented in representatives of these two genera (Miller et al., 2010; Redondo-Nieto et al., 2013; Alvarez et al., 2022). *In vitro* testing of the phosphate solubilization showed that almost all of the *Pseudomonas* and *Burkholderia* isolates have this ability, even in the case of *P. brassicacearum* IT-43CA1 that doesn't have either *gcd* or *gad* genes. The two latter genes encode the production of gluconic and 2-ketogluconic acid, while there are many other acids which are known to be involved in phosphate solubilization (Zaidi et al., 2009). Majority of *Pseudomonas* possess *nirS* or *nirK* genes for denitrification, while *Kosakonia* isolate IT-91MI3 harbors the entire *nif* operon, responsible for the Molybdenum (Mo) - iron (Fe) nitrogenase dependent N fixation, in line with the genomic insight of *K. oryzae* Ola 51T (Li et al., 2017).

With the subsequent screening, which focused on the effect of bacterial VOCs on *F. graminearum* Fg1 growth and the impact of bacterial exudates on fungal conidia germination, selection of potential antagonistic isolates was narrowed further. As a complement to dual-culture assay, these two assays were performed aiming to recruit bacterial strains that can affect different stages of *F. graminearum* Fg1 life cycle (Besset-Manzoni et al., 2019), since this pathogen can persist in soils in the form of mycelium or spores (Goswami and Kistler, 2004). Some antagonistic isolates from non-fungistatic soils can indeed produce VOCs, as *Pseudomonas* strain IT-47CA2 releases HCN *in vitro*, *Pseudomonas* strain IT-194MI4 possesses genes for HCN production, whereas *Burkholderia* strains IT-158MI4 and IT-111MI5 harbor *adh* gene involved in 2,3-butanediol conversion to acetoin. Previous research showed that *Pseudomonas* and *Burkholderia* species can be effective at inhibiting *Fusarium* mycelia development through production of different VOCs (Cordero et al., 2014; Weisskopf, 2014).

Bacterial exudates are rich in secondary metabolites with potential antifungal activity (Xu et al., 2019). A study by de Fátima Dias Diniz et al. (2022) showed that *F. verticillioides* conidia germination was inhibited by cell-free supernatants of bacteria identified as *Achromobacter xylosoxidans*, *P. aeruginosa* and *B. velezensis*, while Wang et al. (2020b) showed that *B. velezensis* cell-free exudates may inhibit germination of *F. graminearum* conidia. In this study, significant inhibition of *F. graminearum* Fg1 conidia germination was achieved in the presence of *P. donghuensis* IT-53CA3 (from fungistatic soil) exudates. Similarly, it has been shown that phenazine-producing *Pseudomonas* may impact the activity of *F. graminearum*-synthesized protein histone acetyltransferase, thus disrupting histone acetylation and fungal conidiation (Chen et al., 2018c). As *P. donghuensis* IT-53CA3 does not possess a gene cluster involved in phenazine production, it might be that other exudates produced by this strain are involved in conidiation inhibition. For example, this strain harbors BGC involved in NRPS-like secondary metabolite synthesis that may be responsible for conidia germination inhibition (Sonkar et al., 2022).

Based on the results of the dual-culture assay, the effect of bacterial VOCs on *F. graminearum* Fg1 growth and the impact of bacterial exudates on conidia germination, bacterial isolates were selected for the *in planta* phytoprotection assay. When *in planta* assay was performed, *B. velezensis* IT-133MI5 and the four *Pseudomonas* strains (including *P. donghuensis* IT-53CA3 from fungistatic soil) limited disease symptoms in wheat. In addition, *B. velezensis* IT-133MI5 inhibited seed germination, which is in line with studies where *Bacillus* sp. X20 was found to inhibit germination of wild oat seeds for 75 % (Li et al., 2021), and *B. megaterium* and *B. circulans* significantly reduced germination of *Orobanche crenata* (Elabaied et al., 2017). Although the four *Pseudomonas* strains from this study harbored a high number of phytobeneficial genes and functions, they resulted in reduced shoot biomass and *Pseudomonas GS-5* IT-194MI4 also gave reduced chlorophyll rate (but improved seed germination), pointing to a trade-off between plant protection and plant growth (Karasov et al., 2017). Trade-off between plant protection and growth is a known phenomenon, where plants aim to use the limited resources in an optimal way (Wang et al., 2021). Reason behind this phenomenon is that activation of defense mechanisms needs a substantial amount of energy and ressources, so under the biotic attack, genes involved in photosynthesis and plant growth are downregulated, while the secretion of defense compounds is increased. Inversely, in certain cases, for example when plants need to reach the light during germination, their growth is faster, at an expense of being more susceptible to different pathogens and pests. All of these are known as 'plant economics' principles, when plants adjust to the outside conditions, trying to balance between defense and growth (He et al., 2022). Among the strains tested in *in planta* assay, the four *Pseudomonas* representatives synthesized siderophores, known for their role in inducing ISR in plants (Bakker et al., 2007). ISR can also be triggered by different VOCs, such as acetoin and 2,3-butanediol (Ryu et al., 2004), whose biosynthetic genes were found in *B. velezensis* IT-133MI5. Genome sequencing and annotation also identified genes whose products have antifungal properties, potentially leading to direct *Fusarium* inhibition. Thus, *P. chlororaphis* IT-48CA2 harbored genes involved in the production of HPR, phenazine (as confirmed by antiSMASH) and pyrrolnitrin. All four *Pseudomonas* contained genes encoding HCN, which has antifungal effects (Ramette et al., 2003), and HCN production was confirmed for all of them but *Pseudomonas GS-5* IT-194MI4. Lytic enzymes, such as proteases, chitinases and cellulases play a key role in antagonism towards *Fusarium* (Rathore et al., 2020). Thus, proteases production *in vitro* was observed in all four *Pseudomonas* and *B. velezensis* IT-133MI5; *P. chlororaphis* IT-48CA2 and *Pseudomonas GS-5* IT-194MI4 had *aprA* genes, responsible for alkaline metalloproteinase production. Chitinase was produced by all four *Pseudomonas* and *B. velezensis* IT-133MI5, while cellulase was produced only by *B. velezensis* IT-133MI5. However, these inconsistent results of *in vitro* and *in planta* experiment were not surprising, as it was already shown that many microbes

that perform well in *in vitro* conditions, fail in greenhouse *in planta* and field experiment (Comby et al., 2017; Besset-Manzoni et al., 2019). But why does this happen?

Most of the phytoprotection studies select microbes based on *in vitro* assays*,* where pathogens are directly inhibited by the antagonistic microbe, but in the system plant-phytopathogen-antagonistic microbe, there are other mechanisms that may take place. For example, it is known that microbes can enhance plant defenses by inducing systemic resistance upon the pathogen attack (Magotra et al., 2016), or can enhance plant fitness, i.e., by increasing the bioavailability of potassium, phosphorus, nitrogen, iron and other essential minerals (Kızılkaya, 2008; Rasouli-Sadaghiani et al., 2014; Dasila et al., 2023). In such a way, interactions that take place when the plant is present in the system are more complex than when interactions are observed solely on the pathogen-microbe level. Thus, choosing microorganism for the plant assay based on one *in vitro* experiment may be challenging and misleading (Besset-Manzoni et al., 2019). Comby et al. (2017) performed a two-fold screening study of wheat endophytes that could potentially protect wheat from FHB, i.e., they performed a classical dual-culture assay, and they checked the ability of endophytes to protect wheat spikelets from disease, and found contrasting results between the two approaches, raising the question of the most suitable screening approach. Nonetheless, factors such as the host plant compatibility, or inoculation method (seed-coating, root-diping, foliar or soil inoculation), largely impact the successs of bacterial inoculants. For example, it is known that some bacteria act as bioherbicides, thus contributing to the reduction of seed germination and plant growth (Fang et al., 2022), while different methods of inoculation may also largely affect the outcome (Stoll et al., 2021). One limiting factor of this study is that soil used for the *in planta* experiment was sterilized and free from other microorganisms that might antagonize the antagonistic bacteria tested, and conditions, i.e., temperature and lighting, were controlled, which is unlike the conditions present in the field. In an ideal case, after the greenhouse *in planta* assay, a field assay should be performed, as it was shown that only few microbes that performed well in *in vitro* and greenhouse *in planta* experiments, were also successful in field conditions, with changing environmental conditions (Pliego et al., 2011). One of the possible solutions to avoid this discrepancy between the different assays, would be to use microbial consortia made from multiple microorganisms with different modes of action (Nadeem et al., 2013; Besset-Manzoni et al., 2018). Although there are pieces of evidence that the consortia activity is higher in the greenhouse *in planta* assay, than in field assays, it still performs better than single-bacteria inoculants (Liu et al., 2023). However, pathway to formulating successful bacterial consortia is time-consuming, as it is required to select bacteria with different modes of action, to verify their compatibility, pathogenicity, as well as their ability to colonize the rhizosphere (Minchev et al., 2021). Finally, as plants may modulate microbial metabolism and the microbial community, there is an urge to better understand plant-microbe interactions and to use that knowledge to formulate consortia with high survival in natural conditions and high plant compatibility (Maciag et al., 2023).

6.3. *Pseudomonas* in suppressive vs. non-suppressive soils

Previous comparisons of soils suppressive vs. non-suppressive to *Fusarium* diseases have revealed differences in the occurrence or prevalence of various taxa (Cha et al., 2016; Siegel-Hertz et al., 2018; Ossowicki et al., 2020; Lv et al., 2023), suggesting that microorganisms associated with suppressive conditions are likely to contribute to plant protection. In this context, this research was focused on *Pseudomonas*, one of the key taxa thought to play a role in disease suppressiveness. Their genetic characteristics enable them to colonize different soils, including disease-suppressive soils (Weller et al., 2007; Kyselková and Moënne-Loccoz, 2012; Santoyo et al., 2012) and they exhibit a wide range of plant-growth promoting and biocontrol properties, such as producing antifungal compounds, competing with pathogens

and triggering ISR in plants (Kloepper et al., 1980; Sneh et al., 1984; Weller et al., 2007; Almario et al., 2013; Vacheron et al., 2016; Legrand et al., 2019; Shen et al., 2022). Shen et al. (2022) suggested that *Pseudomonas* populations might be stimulated in suppressive soils, due to the pathogen pressure and dynamic interactions with the other microbial populations. Additionally, *Pseudomonas* with biocontrol properties have already been isolated from suppressive soils, including *Pseudomonas* sp. Q2-87 (*P. corrugata* subgroup; Weller et al., 2007), isolated from wheat in take-all decline soils, which protects tomato from *F. oxysporum* f. sp*. radicis-lycopersici*, as well as *Pseudomonas* sp. C7 (*P. corrugata* subgroup; Lemanceau and Alabouvette, 1991) isolated from soil suppressive to Fusarium wilt of tomato.

Given the above, the *rpoD* primers of Manriquez (2021) were used for the first time for metabarcoding analysis of *Pseudomonas* populations in suppressive soils. Comparison of soils MI2, MI3, MI4 and MI5 evidenced significant differences in Simpson α-diversity index (which measures both species richness and relative abundance; Hagerty et al., 2020), as it was significantly lower for soil MI4 (non-fungistatic and non-suppressive), compared to the other (suppressive) soils. A higher Simpson index was also evidenced in soils suppressive to wilt disease of banana mediated by *F. oxysporum* f. sp. *cubense* (compared with conducive soils), but this was at the scale of the total bacterial community (Nisrina et al., 2021). *Pseudomonas* taxonomic composition differed between the four MI soils, which is reminiscent of denaturing gradient gel electrophoresis results showing differences in DAPG$^+$ *Pseudomonas* (sub)populations in soils suppressive and non-suppressive to Thielaviopsis black root rot of tobacco (Frapolli et al., 2010).

Further, 406 putative *Pseudomonas* were isolated, from the rhizospheres of wheat plants grown in suppressive and non-suppressive soils MI2, MI3, MI4 and MI5, that had or had not been previously inoculated with *F. graminearum* Fg1. Amplification using the *rpoD* primers, targeting the *rpoD* alleles of bacteria from the *P. fluorescens* group (Frapolli et al., 2007), was successful with 185 of the isolates, while others were identified based on the 16S rRNA gene. Identification based on 16S rRNA gene enabled identification at the genus level, and showed that some of the isolates belonged to genera *Aeromonas*, *Stenotrophomonas*, or *Janthinobacterium* (all members of phylum *Pseudomonadota*), yielding 52 additional *Pseudomonas*. Significant number of isolates that do not belong to the *P. fluorescens* group was obtained due to the fact that the isolation media used (i.e., King's B agar) is not strictly selective for *Pseudomonas* (Sands and Rovira, 1970; Johnsen and Nielsen, 1999). Although King's B was supplemented with different antibiotics, i.e., with ampicillin, chloramphenicol (acting against Gram positive and some Gram negative bacteria) and cycloheximide (acting against eukaryots, including fungi), this was still not enough to obtain only *Pseudomonas* isolates.

Altogether 29 *Pseudomonas* were chosen for the whole-genome sequencing (on the basis of combination of isolates from four different MI soils with and without *F. graminearum* Fg1 inoculation), and they belonged to the *P. fluorescens* group, i.e., *P. fluorescens*, *P. kielensis*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata* and *P. chlororaphis* subgroups. Distribution of these 29 sequenced *Pseudomonas* taxa was rather soil-specific, as six out of seven species from MI2 were found only in soil MI2, two out of four species only in soil MI3, seven out of nine species only in soil MI4, and three out of six species only in soil MI5, which is largely in line with *rpoD* metabarcoding data from this study. Whole-genome sequencing of the 29 *Pseudomonas* and dDDH values (obtaining values below 70%, a threshold recognized for species delineation (Chun et al., 2018)) revealed as many as 16 novel *Pseudomonas* genomospecies. During this part of research, two newly identified *Pseudomonas* species, and their morphological, genomic, biochemical and physiological features were fully described - names *P. serbica* (for strains IT-P366^T and IT-194P) and *P. serboccidentalis* (for strains IT-P374^T and IT-215P) were proposed (Oren and Goker, 2023; Todorović et al., 2023a), following the guidelines of International Code of Nomenclature of Prokaryotes (Oren et al.,

2003). When it comes to genomic features of the newly identified species, strains of *P. serbica* possess certain specificities. A megaplasmid of 1,059,298 bp identified in strain IT-P366^T is absent from the genome of the second strain IT-194P of the proposed species *P. serbica*. The presence of this plasmid partly explains the large size difference between the genomes of the two strains (792,935 bp). Megaplasmids are rare in *Pseudomonas*, but they can allow the host cell to expand its specific niche (Kuepper et al., 2015; Purtschert-Montenegro et al., 2022). Thus, this megaplasmid hosts an operon for the synthesis of a type IVB secretion system (Dot/Icm family; Costa et al., 2021), whose homolog was recently described to be involved in the biocontrol of a bacterial pathogen (Purtschert-Montenegro et al., 2022), a chemotaxis operon and a flagellum synthesis operon. These functions represent an addition to the core species functions encoded in the chromosome of *P. serbica*, which contains another flagellum synthesis operon (identical to the one of IT-194P). Future functional studies involving the removal of this megaplasmid from strain IT-P366, as well as its transfection into other strains (such as IT-194P), are needed in understanding its role in different plant-microbe interactions and adaptation to the surrouding. Besides these two formally described species, a substantial number of potentially biocontrol-relevant species uncovered during the course of this thesis, remain undescribed, and require future steps in order to describe them.

Pseudomonas from the fluorescent group have been extensively studied in the case of Fusarium wilt, notably in southern France (Alabouvette, 1986) and in California (Scher and Baker, 1980), where main mechanisms underlying this disease suppression were found to be synthesis of phenazine (Mazurier et al., 2009) and competition for iron (Scher and Baker, 1980). Notably, the four *P. chlororaphis* strains, which originated from MI3 and MI5 soils (both suppressive to *F. graminearum* disease), harbored as many as 11 to 13 genes involved in biocontrol or plant-growth promotion. This may reflect the taxonomy rather than the soil origin of the strains, as the ability of *P. chlororaphis* to produce compounds with antimicrobial activity (Arseneault and Filion, 2016) and protect plant is well documented (Raio and Puopolo, 2021). *P. brassicacearum* IT-228P was the only isolate with potential to produce DAPG, a prominent biocontrol metabolite in several types of suppressive soils (Frapolli et al., 2010; Weller et al., 2007). Characterization of activities *in vitro* revealed a wide distribution among the 29 strains, with again taxonomic particularities (with up to 13 phytobeneficial functions per strain). Nevertheless, when comparing the soils of origin, there was a rather even distribution of plant-growth promoting and biocontrol properties (both genetic and phenotypic) among the strains, regardless of the experimental conditions (i.e., soil suppressiveness/fungistasis status and inoculation status). Although plant protection that takes place in suppressive soils may be a result of action of one or a few microbial populations, it may be that other microbial community members have an important role on the former, i.e., that they influence their root colonization or biocontrol gene expression (Kyselková and Moënne-Loccoz, 2012). For example, DAPG-producing *P. protegens* strains are found in both soils suppressive and non-suppressive to black root-rot disease of tobacco, but their phytoprotective capacities differ. It was shown that this was due to the presence of iron-releasing minerals in suppressive soils, that alter iron bioavailability and positively impact the expression of DAPG genes in suppressive soils. This was confirmed by adding iron to non-suppressive soils, which resulted in an enhanced expression of DAPG genes in these soils (Almario et al., 2013). Therefore, the next stage in defining particularities between disease suppressive and non-suppressive soils would be to assess the levels of expression of biocontrol genes. Another important point is that a population of a biocontrol strain has to achieve a certain threshold in order to achieve phytoprotection (Weller et al., 2007). For example, it is known that non-pathogenic *F. oxysporum* Fo47 is needed in concentrations 10 to 10^2 times higher than the phytopathogen itself, in order to suppress the pathogenic *F. oxysporum* (Fravel et al., 2003). Similarly, it has been observed that *P. defensor* WCS374 has the ability to suppress Fusarium wilt, but only if present at $\sim 10^5$ CFU per g of root

(Raaijmakers et al., 1995). Therefore, it would be useful to assess the relative abundance of different *Pseudomonas* genotypes. Overall, analysis of microbial communities in soils of contrasted suppressiveness status seems as a promising approach in an attempt to identify taxa that are more abundant, or whose genes are more expressed in suppressive soils, as those taxa might represent potential plant-protecting microbes (Benítez and McSpadden Gardener, 2009; Pliego et al., 2011).

For the *in planta* assay, four *P. chlororaphis* strains and one *P. brassicacearum* strain were chosen. Although they harbored a high number of phytobeneficial genes and functions, none of the *Pseudomonas* isolates showed phytoprotective effects on wheat plants, in the presence of *F. graminearum* Fg1. As previously outlined by Vacheron et al. (2016), fluorescent *Pseudomonas* strains with up to five plant-beneficial properties, are favored in the rhizosphere of maize plants, while strains with many phytobeneficial properties may have shorter survival in the rhizosphere (Weller, 1988). It is also well known that certain *Pseudomonas* strains may act as herbicides and inhibit plant growth and development, through overproduction of auxins, exopolysaccharides, phytotoxic metabolites or through some unknown mechanisms (Fang et al., 2022). IAA has a positive impact on plant growth at appropriate concentrations, while overproduction might be deleterious for the plant (Fang et al., 2022). Here, all *Pseudomonas* strains were producing IAA, except *P. chlororaphis* IT-342P, which harbored *iaaMH* genes, but did not produce IAA in *in vitro* conditions. Similarly, all of these *Pseudomonas* strains are HCN-producers, and besides the fact that HCN contributes to pathogen suppression, its phytotoxic effects were also demonstrated before (Kremer et al., 2006). For example, HCN-producing *P. fluorescens* S241 inhibited bean and lettuce growth via its cyanide producing ability (Alström and Burns, 1989). Moreover, *P. fluorescens* D7 is a registered bioherbicide acting on *Bromus tectorum* (Tekiela, 2020), while P. *kilonensis*/*brassicacearum* G11 reduced growth and root length of *Echinochloa crus-galli* (Zeller et al., 2007). Certain PGPR may also have herbicidal activity (Fang et al., 2022), as it was shown with *P. fluorescens* strain Bf7-9, that had a positive impact on growth of faba bean, and at the same time reduced the emergence of plants *Orobanche foetida* and *O. crenata* (Zermane et al., 2007). Similarly, *P. marginalis* Nc1-2 positively impacted faba bean growth, but it reduced the emergence of *O. crenata* (Zermane et al., 2007). Such dual function of certain bacterial strains is a result of different specificity towards different plant species, and it is an important trait to consider when aiming to select a potent biocontrol agent (Mejri et al., 2010).

6.4. Secondary metabolites in soil suppressiveness to *Fusarium graminearum*

Microorganisms are able to produce a wide variety of secondary metabolites, including antibiotics and VOCs, that are not involved in primary metabolism, but, rather, help microbes to harvest nutrients and to interact and communicate with other microorganisms, including competitors and symbionts (Macheleidt et al., 2016). Secondary metabolites have small molecular weight and they are very structurally heterogeneous, with a vast potential still being unraveled (Keswani et al., 2020). Several identified secondary metabolites have already been linked to disease suppressiveness, such as the production of thiopeptide by *Streptomyces* (Cha et al., 2016), phenazines by *Pseudomonas* (Mazurier et al., 2009), and production of iturin C, bacillomycin, fengycin by *B. licheniformis* (Yadav et al., 2021) in the case of Fusarium wilt, as well as the production of DAPG by *Pseudomonas* in the case of take-all disease of barley and wheat (Weller et al., 2007). Here, several putative BGCs have been identified in both biocontrol and *Pseudomonas* strains, that could be potentially involved in the suppression of *F. graminearum* and damping-off disease. In addition, several biocontrol and *Pseudomonas* strains produced VOCs with antagonistic properties against *F. graminearum,* but this would require further identification of exact VOCs that act antagonistically.

The BGCs found in strains from this study mostly encoded for siderophores, lipopeptides and polyketides, groups of metabolites that are widely known for their antifungal properties (Chen et al., 2009; Esmaeel et al., 2016). For example, strain *Brevibacillus GS-3* IT-7CA2 harbors BGCs involved in the production of peptides, such as edeine, gramicidin and tyrocidin (Yang and Yousef, 2018), which have strong antifungal effects and may have contributed to *F. graminearum* inhibition by this strain in a dual-culture assay. Strain *B. velezensis* IT-133MI5 harbors BGCs involved in production of lipopeptides mycosubtilin, plipastatin, surfactin (Ongena and Jacques, 2008), polyketide difficidin (Rabbee et al., 2019), peptide mersacidin (Emam and Dunlap, 2020) and peptide bacilysin (Mateus et al., 2021), all with potential antimicrobial activity. *P. soli* IT-47CA2 has BGC involved in the production of lipopetide xantholysin, with a confirmed role in the biofilm formation and antifungal activity against various fungi, including *F. graminearum* (Li et al., 2013). When it comes to specificities of newly described *Pseudomonas* species, antiSMASH showed that the proposed species *P. serbica* (strains IT-P366^T and IT-194P) harbor species-specific gene clusters involved in steroid degradation, previously described in *Comamonas testosteroni* Y1, isolated from activated sludge (Li et al., 2022) and in several manure-borne proteobacterial species, but not in *Pseudomonas* (Yang et al., 2011). Besides, strains P366^T and IT-194P contain genes *bcsABGQZ*, known to be involved in the synthesis of cellulose, which is contributing to biofilm formation and promoting the epiphytic lifestyle of *P. syringae* (Arrebola et al., 2015). *P. serboccidentalis* (strains IT-P374^T and IT-215P) hosts an operon for the synthesis of a type VI secretion system, which was recently confirmed as detrimental for fungal cells (Trunk et al., 2018). Similarly to *P. serboccidentalis*, *P. brassicacearum* IT-228P hosts an operon for T6SS, and also operon for T3SS, already reported for this species (Ortet et al., 2011). The three *P. chlororaphis* strains (i.e., IT-196P, IT-201P and IT-373P) harbor BGC involved in production of lipopeptide massetolide A, previously documented in *P. fluorescens* SS101, and with biocontrol activity against *Phytophthora infestans*, causal agent of late blight in tomato (Tran et al., 2007). These findings suggest that strains from this study could play an important role in sustainable agriculture if used as biocontrol agents.

However, presence of certain BGC in the bacterial genome does not necessarily mean that the corresponding metabolite is indeed synthesized and excreted in the rhizosphere, moreover as the BGC expression is often determined by the surrounding abiotic and biotic conditions (Dastogeer et al., 2020). Therefore, tools such as transcriptomic, proteomic and metabolomics studies, as well as the use of reporter genes, such as the *Green Fluorescent Protein* (GFP), can help in elucidating the production of secondary metabolites encoded in the bacterial genome (Kiely et al., 2006; Barret et al., 2009; Mavrodi et al., 2021). For example, genome mining revealed that *Bacillus cabrialesii* TE3T contains BGCs coding for the production of several secondary metabolites, while the metabolomic techniques demonstrated that only surfactin, fengycin, and rhizocticin A are indeed produced and have antifungal activity against phytopathogen *Bipolaris sorokiniana*, a causal agent of spot blotch disease of durum wheat (*Triticum turgidum* L. subsp. *durum*) (Villa-Rodriguez et al., 2021). Another issue is that high genetic diversity of BGCs in living organisms, together with limited verified databases that could help in encoding their exact functions, leaves only the possibility of assuming their precise functions. In order to verify if certain BGCs from isolated bacteria indeed play a role in disease suppression, a site-directed mutagenesis is required, so that the antifungal properties of wild type and mutated strains could be compared (Wang et al., 2020). Similar research has already been conducted by Mendes et al. (2011), when they performed transposon mutagenesis on a gene cluster encoding thanamycin synthesis in strain *Pseudomonas* sp. SH-C52, obtained from suppressive soils, and showed that the mutant had the ability to colonize the rhizosphere of sugar beet seedlings, but could not protect it from *R. solani* infection like the wild type. In a similar manner, potential biocontrol success of

Pseudomonas strains from this study, via BGCs discovered in their genomes, would have to be validated and their function possibly linked to fungistatic or suppressive soil status.

7. CONCLUSIONS

Overall, the research conducted during this PhD dissertation presents the first screening of fungistasis to *F. graminearum* across 26 contrasting fields in the Republic of Serbia and screening of suppressiveness to *F. graminearum* damping-off disease of wheat at the same time. In general, this PhD dissertation outlines the microbial nature of fungistatic and suppressive soils, with dynamic interactions taking place between numerous actors in the rhizosphere, and yields the following conclusions:

1. Soils fungistatic to phytopathogen *F. graminearum* Fg1 were identified for the first time in Serbia. Out of 26 sampled agricultural fields from northern and western/central regions of Serbia, 38% were fungistatic (all from western/central Serbia), while 62% were non-fungistatic. Microbial, rather than physicochemical, basis of fungistatic soils was confirmed, since the fungistatic property was lost following the sterilization of soils.
2. Manure was identified as a significant farming practice promoting soil fungistasis towards the wheat pathogen *F. graminearum* Fg1 at locations near Mionica (fields MI2, MI3, MI4 and MI5), and a similar trend was observed for the soils near Čačak.
3. In soils where manure is of particular importance for fungistasis (near Mionica), two fungistatic soils, but also one of the non-fungistatic, were also suppressive to *F. graminearum* Fg1 damping-off disease in wheat. This was the first time that suppressive soils were documented in Serbia. This work corroborates the idea that fungistasis is not a prerequisite for suppressiveness and that plant protection by microorganisms occurs through fungistasis-dependent mechanisms (direct pathogen inhibition in soils), as well as via plant-dependent mechanisms (induction of ISR in plants or through rhizosphere interactions).
4. It was shown that the rhizospheres of wheat grown in suppressive and non-suppressive soils shared the main prokaryotic and fungal phyla, and the majority of the most abundant taxa, yet several taxa were specific for each soil.
5. In exploring the usefulness of fungistatic soils as sources of antagonistic strains, 23 bacterial isolates (originating from fungistatic and non-fungistatic soils) that inhibited growth of *F. graminearum* Fg1 in *in vitro* conditions, were uncovered and selected. Genome sequencing of these 23 strains revealed that the 10 strains from fungistatic soils belonged to the phyla *Pseudomonadota* (three strains from the genus *Pseudomonas* and one from the genus *Kosakonia*) or *Bacillota* (four strains from the genus *Bacillus* and two from the genus *Priestia*). The 13 strains from non-fungistatic soils belonged to the phyla *Pseudomonadota* (seven from *Pseudomonas* and two from *Burkholderia*), *Bacillota* (two from *Bacillus* and one from *Brevibacillus*), as well as *Bacteroidota* (one from *Chryseobacterium*). Altogether, most representatives from both fungistatic and non-fungistatic soils belonged to phyla *Bacillota* and *Pseudomonadota*, although to different species. Among these, eight novel genomospecies were also revealed.
6. Genomic and functional characterization of antagonistic strains revealed that their biocontrol potential is not related to the fungistasis-related soil status, but, rather, to the taxonomy. These results outline the fact that not only fungistatic soils are good sources of potential biocontrol agents, and that relative abundance of biocontrol strains or level of expression of biocontrol gene(s) may contribute to the formation of fungistatic soils.
7. *P. chlororaphis* strains IT-51CA3, IT-162MI3 (originating from fungistatic soils) and IT-48CA2 (originating from a non-fungistatic soil), harbored genes that encode antifungal metabolites, such as phenazine, HPR, pyrrolnitrin and HCN, while *P. brassicacearum* harbored genes for DAPG production, making them ideal candidates for biocontrol.
8. *F. graminearum* Fg1 conidia germination was inhibited by exudates of *P. donghuensis* IT-53CA3 (from fungistatic soil) by 75%, while *P. soli* IT-47CA2 and *B. ambifaria* IT-158MI4

(both from non-fungistatic soils) inhibited mycelial growth of *F. graminearum* Fg1 by VOCs for more than 40%. On top of the ability of the aforementioned strains to inhibit *F. graminearum* Fg1 in confrontation assay, they could also inhibit *F. graminearum* Fg1 via exudates targeting conidia germination or VOCs preventing mycelial growth, making them good candidates for fungal inhibition in different phases of its lifecycle.

9. Antagonistic rhizosphere strain *Pseudomonas GS-5* IT-194MI4 (from non-fungistatic soil) enhanced wheat germination for 25% and conferred significant protection from crown-rot disease, compared to the control inoculated with *F. graminearum* Fg1, but at the expense of shoot biomass and chlorophyll rate. This strain could potentially be used to enhance wheat germination and offer plant protection in *F. graminearum* affected areas, or it could be used in consortia that would act synergistically and offer both plant protection and enhanced wheat yield, but this requires further research.
10. Metabarcoding analysis, based on *rpoD* gene sequence, for the first time, showed that fluorescent *Pseudomonas* community differs between suppressive and non-suppressive soils.
11. The genome sequencing of 29 *Pseudomonas* from suppressive and non-suppressive soils, enabled their affiliation to seven out of 11 subgroups of the *P. fluorescens* group, i.e., the subgroups *P. fluorescens*, *P. kielensis*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata* and *P. chlororaphis*. Among these, 16 novel genomospecies were revealed.
12. Two novel species were described genomicaly and phenotypically, proposing the names *P. serbica* (for strains IT-P366^T and IT-194P) and *P. serboccidentalis* (for strains T-P374^T and IT-215P). These analyses showed that these two novel species might have a potential role in biocontrol, however, additional tests including pathogenicity tests, *in planta* assay and field trials would have to be performed.
13. Genomic and functional particularities of *Pseudomonas* strains from suppressive and non-suppressive soils were compared, revealing similar profiles in both soils. This, again, outlines the fact that the relative abundance of *Pseudomonas* strains or levels of expression of biocontrol gene(s) potentially contribute to the formation of suppressive soils.
14. *F. graminearum* Fg1 conidia germination was inhibited by two *Pseudomonas* strains for more that 10%, and three *Pseudomonas* strains had the ability to inhibit growth of *F. graminearum* Fg1 for more than 20% by VOCs. These strains can potentially be used to inhibit fungal development in different phases of its lifecycle.
15. Among all tested *Pseudomonas*, none contributed to wheat phytoprotection from crown rot disease, while *P. chlororaphis* IT-373P (from suppressive soil MI5) and *P. brassicacearum* IT-228P (from non-suppressive soil MI4) were found to contribute to wheat germination, at an expense of shoot biomass and chlorophyll rate. However, these strains should be tested with different plant species or using different inoculation methods, and this may lead to their better success. Moreover, strains that contributed to even smaller wheat germination rate, could potentially be used as bioherbicides.
16. In genomes of both antagonistic and *Pseudomonas* strains, a substantial number of BGCs encoding for production of secondary metabolites, such as siderophores, lipopeptides and polyketides were found, making them promising candidates for biocontrol, but this needs further verification. In genomes of both antagonistic and *Pseudomonas* strains, type III, IV and VI secretion systems were detected, and this may be significant in bacterial-fungal interactions, modulation of plant immunity and biocontrol.

In conclusion, as this research represents the first screening of soils suppressive to *F. graminearum* disease (not just in Serbia, but worldwide), the data obtained may serve as a foundation for further research on soils suppressive to *F. graminearum* diseases and a base for rhizosphere microbiome studies, adding up to the research already conducted on soils

suppressive to *Fusarium* diseases in other parts of the world, in different climatic conditions and with different agricultural practices. Furthermore, an immensely important result of this research is the formation of a collection of profoundly characterized bacterial strains that have a vast potential to be used in sustainable agriculture. Given that here, fungistasis and suppressiveness assays were performed at locations of contrasting landscape, soil types, and agricultural practices, this approach should be implemented in future research, as this might help decipher the occurrence patterns of the mentioned phenomena. Metatranscriptomics and metabolomics will be usefull in future research on suppressive soils, in order to check for differences in plants grown in these, compared to non-suppressive soils. Additionally, it would be useful to perform a field study with the potential biocontrol agent, *Pseudomonas GS-5* IT-194MI4, to check for its viability in natural environment, as well as to try different methods of inoculation for other strains. This might help in targeting actors that may be used in designing a consortium able to protect plants from *F. graminearum* diseases. Another future direction would be to describe species, newly found during the course of this thesis. Since there were no particularities that made a distinction between *Pseudomonas* found in suppressive vs. non-suppressive soils, it would be of interest to examine the expression of genes involved in biocontrol or to check for the relative abundance of different *Pseudomonas* genotypes. This study also pinpointed the metabolic potential of wheat microbiome to produce various metabolites, including secondary metabolites, that may be important in soil suppressiveness to *F. graminearum* diseases, but this aspect needs further validation. In general, deep understanding of mechanisms underlying soil suppressiveness may help in inducing suppressiveness at sites where crops are severely attacked by *F. graminearum*, as well as by other detrimental pathogens. Finally, research such as this one represents a small, but very significant step towards decreased crop and economical lossess, as well as the protection of human and animal health compromised by *Fusarium* mycotoxins.

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9. SUPPLEMENTARY MATERIAL

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GENERAL INTRODUCTION

Soil is a dynamic ecosystem, a complex mixture of inorganic and organic matter, inhabited by a diverse array of microorganisms, plants and animals (Tešić & Todorović, 1988; Chandrashekara et al., 2012). It is widely recognized that soil has a crucial role in crop productivity and health, serving as a fertile ground for microbial collaboration and a battleground for dynamic interactions between the soil-dwelling microorganisms and plants. Therefore, soil forms the foundation of sustainable agriculture (Raaijmakers et al., 2009). In 1987, sustainable development was defined in the report of the World Commission on Environment and Development as "development that meets the needs of the present without compromising the ability of future generations to meet their own needs" (World Commission on Environment and Development, 1987). However, agricultural production has been facing serious challenges in recent decades. The prevailing agricultural practices, which involve the use of agrochemicals, pose a significant threat to the environment and contribute to soil pollution. Furthermore, accelerated urbanization and industrialization have resulted in a substantial reduction in agricultural areas (Saeed et al., 2021). The excessive use of agrochemicals further exacerbates climate change by contributing to the greenhouse effect through the emission of harmful gases and the deposition of toxic components into the soil (Koli et al., 2019). These issues emphasize the need to adopt alternative approaches in agriculture that prioritize environmental sustainability and food security. It is crucial to protect crops from phytopathogens and to increase crop yields within the existing agricultural areas. Consequently, research on plants rhizospheres, which represent an ecological niche for numerous beneficial microorganisms, may offer insights on how to mitigate the consequences of intensive agriculture.

RHIZOSPHERE AND RHIZOSPHERE INTERACTIONS

The rhizosphere, a narrow zone of soil adjacent to the plant's roots, serves as a habitat for a diverse array of phytopathogens and beneficial microorganisms, which interact with each other and are directly influenced by the plant roots' exudates (Figure 1). Bacteria are the most abundant inhabitants of the rhizosphere, as approximately 10^8–10^{12} bacterial cells can be found in 1 gram of rhizosphere soil (Kennedy & De Luna, 2005).

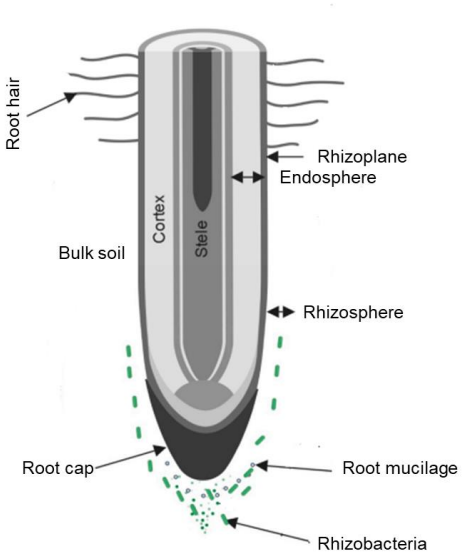

Figure 1. Scheme of microbe-inhabited soil and root compartments, adapted from Hassan et al. (2019).

The "rhizosphere effect", initially described by Hiltner in 1904 (Hiltner, 1904), explains that plant exudates attract a multitude of soil microorganisms, thereby increasing their abundance and activity in the rhizosphere. Plant roots directly release approximately 40% of all photosynthates into the rhizosphere. In addition to carbon compounds, which serve as a nutrient-rich source for the microbial community, plant roots secrete attractants that are recognized by microorganisms, initiating root colonization. This process of plant root colonization plays a crucial role in: (i) facilitating positive interactions, such as communication between plant roots and beneficial microorganisms, (ii) enabling negative interactions, such as root infection by phytopathogens, and (iii) establishing neutral interactions that do not impact either participant (Bais et al., 2006). Given that both phytopathogens and beneficial microorganisms coexist in the rhizosphere, their interactions significantly influence crop productivity and health (Jayaraman et al., 2021).

Positive interactions in the rhizosphere encompass associations with mycorrhizal fungi, rhizobia, and the colonization of plant roots by phytobeneficial bacteria with biocontrol and/or plant growth-promoting properties (Jamil et al., 2022). These phytobeneficial bacteria, referred to as *Plant Growth-Promoting Rhizobacteria* (PGPR), exert numerous beneficial effects on the plant through direct and indirect mechanisms. They contribute to protection from biotic and abiotic stressors, enhance seed germination, and promote root and shoot growth (Glick, 2012).

Direct mechanisms employed by beneficial rhizobacteria involve reducing the harmful effects of phytopathogens by affecting their growth/survival. These mechanisms include: (i) antagonism based on the production of different metabolites, (ii) competition with the pathogens for space and nutrients, and (iii) hyperparasitism (Nguvo & Gao, 2019; Morimura et al., 2020). In addition to these mechanisms that affect the pathogen directly, indirect mechanisms, which are mediated by the plant, come into play. These include induction of plant resistance (ISR), as well as mechanisms that increase plant fitness, making it less susceptible to pathogen attack, such as (i) increasing the solubilization of phosphate and nitrogen fixation, (ii) production of siderophores that enable better absorption of iron, (iii) production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, thus lowering ethylene levels in plants, as well as (iv) production of phytohormones (Figure 2). Through these indirect mechanisms, beneficial bacteria assist in providing plants with essential nutrients such as phosphorus, nitrogen and iron, and alter levels of phytohormones in plants, thereby mitigating the detrimental effects of environmental stressors (Glick, 2012).

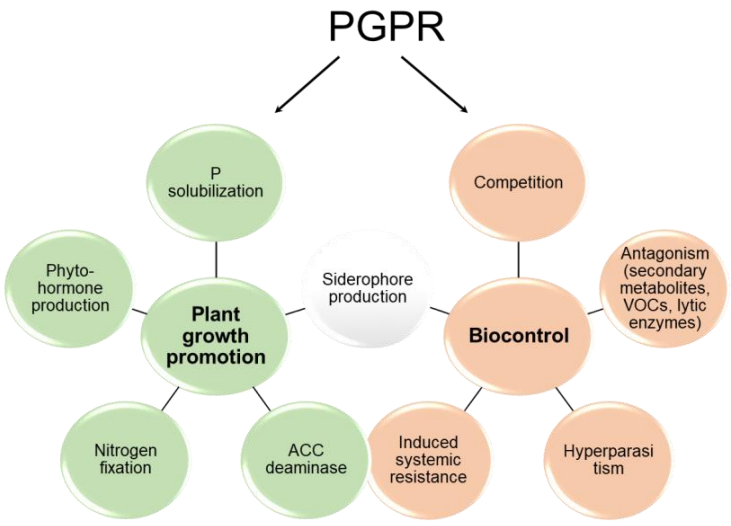

Figure 2. Schematic representation of PGPR plant growth promoting and biocontrol mechanisms.

On the other hand, negative interactions encompass the infection of plant roots with pathogenic bacteria, oomycetes or fungi. The outcome of the pathogen attack is directly determined by the defense capacities of both the plant and the microbial community (Berendsen et al., 2012; Vandenkoornhuyse et al., 2015). If the pathogen manages to overcome this barrier, the infection cycle can commence.

DISEASE-SUPPRESSIVE SOILS

The effect of plant-protecting soil microbiota on plant health is of particular interest in the case of disease-suppressive soils. In these soils, despite the presence of the phytopathogen in the soil, the host plant, and favorable environmental conditions (Baker & Cook, 1974), the disease does not occur, or occurs but is limited. Disease-suppressive soils serve as a model of microbiologically mediated, environmentally acceptable, and efficient methods of protecting plants from phytopathogenic infections. Suppressive soils represent a reservoir of beneficial microorganisms, that can provide effective plant protection against various soil-borne phytopathogens through different modes of action (Morimura et al., 2020). General disease suppression is related to the total soil microbial activity that is restricting growth or survival of multiple pathogens, and is usually taking place in bulk soil (Termorshuizen & Jeger, 2008). If the fungal propagules in soil are affected, through competition with the soil microbiota and excretion of antagonistic compounds, this phenomenon is referred to as fungistasis (Garbeva et al., 2011). Contrarily, specific disease suppression refers to the suppression of specific pathogen-caused disease (not the pathogen), it is related to the activity of one or several specific microbial populations, and usually takes place in the rhizosphere (Termorshuizen & Jeger, 2008).

The biocontrol potential of suppressive soils is of great importance when considering phytopathogens such as mycotoxicogenic *Fusarium graminearum*, which is causing increasing damage to crops in the ongoing climate change. While disease suppressive soils specifically targeting *F. graminearum* have not been documented yet, soils suppressive to diseases caused by other *Fusarium* species in various crops have been identified. It has been shown that representatives of different bacterial groups carry out functions that contribute to the suppression of *Fusarium*-caused diseases (Sneh et al., 1984; Cha et al., 2016; Yadav et al., 2021). For instance, species from the genera *Bacillus*, *Brevibacillus, Burkholderia, Chryseobacterium* and *Kosakonia* are well-known for their role in the suppression of *Fusarium* – caused diseases through various direct biocontrol mechanisms (Tyc et al., 2015; Chen et al., 2018; Johnson et al., 2020; Xu et al., 2020; Singh et al., 2021). Furthermore, some of these bacteria may also exhibit plant-growth promoting properties, such as phosphorus solubilization, nitrogen fixation, production of phytohormones or ACC deaminase production, thereby facilitating the plant growth (Ahemad & Kibret, 2014). Besides before mentioned genera, it is known that fluorescent *Pseudomonas* species have an important role in the rhizosphere and in suppressive soils. These species exhibit a wide range of phytobeneficial functions that can contribute to the inactivation or inhibition of *Fusarium* growth, as well as to the promotion of plant growth (Kloepper et al., 1980; Vacheron et al., 2016; Legrand et al., 2019).

Besides these specific bacterial groups that are affecting pathogen and disease development, it was shown that higher functional and genetic diversity of the whole microbial community in soil positively contribute to soil suppressiveness (Jayaraman et al., 2021). This diversity is determined by the plant species, i.e., plant rhizodeposition and root exudates, and may be modified by certain agricultural practices (Termorshuizen & Jeger, 2008). Different organic amendments, that are serving as a source of nutrients, are often used to promote soil health as they may stimulate soil microbiota (Mousa & Raizada, 2016). For example, it was

shown that compost amendments enhance soil suppressiveness to *Rhizoctonia solani* diseases through stimulation of soil microbiota, and impact on its activity, structure and density (Pérez-Piqueres et al., 2006). Composts may contain the entire microbial consortia, rather than few microbial populations, thereby acting via several mechanisms contributing to soil suppressiveness (Jayaraman et al., 2021). Therefore, adoption of agricultural practices such as the addition of organic amendments or composts, may be used to manipulate soil microbiome and to increase soil suppressiveness to phytopathogens (De Corato, 2020).

THE IMPORTANCE OF *PSEUDOMONAS* IN BIOLOGICAL CONTROL AND PLANT-GROWTH PROMOTION

The proteobacterial genus *Pseudomonas* consists of species with versatile metabolism and physiology, colonizing various aquatic, terrestrial and biotic environments. *Pseudomonas* species display different lifestyles – some are opportunistic human, insect or plant pathogens, some can be used in bioremediation, while others can act as PGPR by providing phytostimulation and/or phytoprotection functions (Silby et al., 2011). Within the *Pseudomonas* genus, the *P. fluorescens* group is the most diverse and complex, usually subdivided into subgroups, represented by the species *P. fluorescens*, *P. fragi*, *P. gessardii*, *P. mandelii*, *P. koreensis*, *P. jessenii*, *P. asplenii*, *P. corrugata*, *P. chlororaphis, P. kielensis* and *P. protegens* (Figure 3; Garrido-Sanz et al., 2016; Hesse et al., 2018; Girard et al., 2021). While some phytopathogens are found within the *P. fluorescens* group (such as *P. corrugata* or *P. mediterranea*; Trantas et al., 2015), it mostly includes various phytobeneficial species.

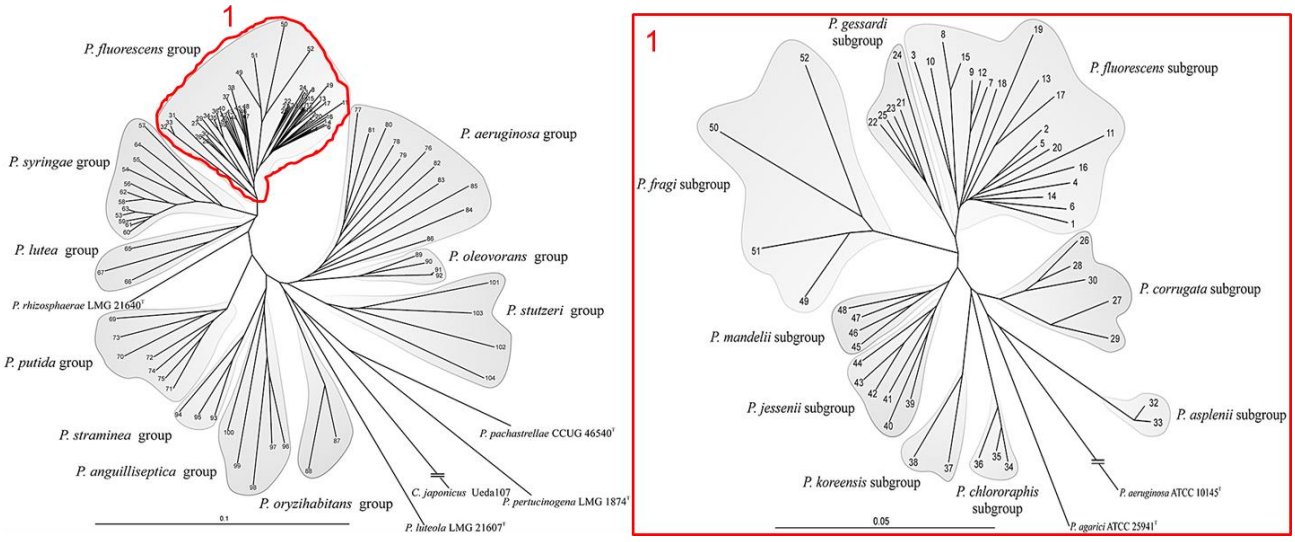

Figure 3. Phylogenetic tree of *Pseudomonas* genus (left) and phylogenetic tree of *Pseudomonas fluorescens* group (right). Trees were constructed based on the concatenated partial sequences of the 16S rRNA gene *rrs*, *gyrB*, *rpoB* and *rpoD* genes. Taken from Mulet et al. (2010).

P. fluorescens group contains species with various PGPR properties, either phytostimulative or phytoprotective, and as such, species of this group have an important role in the rhizosphere (Loper et al., 2012; Sarma et al., 2014; Vacheron et al., 2016). It is known that certain species within this group have the ability to induce ISR in plants and produce a wide range of antifungal substances that can inactivate or inhibit *Fusarium* growth (Vacheron et al., 2016). These antifungal substances encompass antimicrobial secondary metabolites such as pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), phenazine, 2-hexyl-5-

propyl-alkylresorcinol (HPR) or hydrogen cyanide (HCN), as well as lytic enzymes with biocontrol potential like chitinases, cellulases or proteases (Nowak-Thompson et al., 2003; Loper et al., 2012; Sarma et al., 2014; Vacheron et al., 2016; Kumar et al., 2017), which can directly inhibit pathogens. *Pseudomonas* can elicit ISR in plants by producing lipopolysaccharides or flagella, DAPG or siderophores (Bakker et al., 2007). This diversity of modes of action has made species of the *P. fluorescens* group one of the most promising candidates for biological control since the 1970s (Weller et al., 2007). Indeed, fluorescent *Pseudomonas* with biocontrol properties, isolated from soils suppressive to take-all disease of wheat or barley, caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Cook & Rovira, 1976) or soils suppressive to *T. basicola*-mediated black root rot of tobacco (Stutz et al., 1986), have been shown to effectively protect plants from disease (Almario et al., 2014).

In soils suppressive to *F. oxysporum* in Salinas Valley, it was shown that this suppressiveness is attributed to the presence of siderophore-producing, fluorescent *Pseudomonas*, which are more competitive and can complex iron faster than the pathogen (Kloepper et al., 1980; Sneh et al., 1984). In China, *Pseudomonas* strains present in soils suppressive to *F. oxysporum*, induced ISR in banana by increasing levels of jasmonate and salicylic acid, as well as enhancing the activity of polyphenol oxidase (Lv et al., 2023). Besides their phytoprotective role, species of the *P. fluorescens* group are capable of modulating plant growth by producing phytohormones (Vacheron et al., 2016), solubilizing phosphates (Meyer et al., 2010), carrying out denitrification (Almeida et al., 1995) and producing ACC deaminase (Glick et al., 1998; Prigent-Combaret et al., 2008). As a result, fluorescent *Pseudomonas* species have been extensively studied as PGPRs due to their ability to promote plant health through both direct and indirect mechanisms (David et al., 2018). Genome analysis plays a crucial role in uncovering various modes of action employed by these bacteria, as it allows for the characterization of biocontrol and plant growth-promoting functional traits (Van Elsas et al., 2008). Furthermore, genome comparisons enable accurate affiliation of bacterial species, with the help of tools such as Type Strain Genome Server (TYGS; Meier-Kolthoff & Göker, 2019; Meier-Kolthoff et al., 2022) or Average Nucleotide Identity (ANI) calculations (Chun et al., 2018). It has also been observed that certain *Pseudomonas* possess effectors secretion systems, such as type III (T3SS), type IV (T4SS) and type VI (T6SS) secretion systems which are located on the bacterial cell membranes and enable secretion of various compounds (Loper et al., 2012). T3SS is found in numerous Gram-negative species, including certain non-pathogenic *Pseudomonas*, and it can modulate plant immunity (Mavrodi et al., 2011) and enhance the phytoprotective properties of these bacteria (Rezzonico et al., 2005; Marchi et al., 2013). T4SS is found in many bacterial species, and it has been shown to act as a defense mechanism in *P. putida*, protecting tomato plants from pathogenic *Ralstonia solanacearum* (Purtschert-Montenegro et al., 2022). T6SS, which is present in various *Pseudomonas* species, including *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Pseudomonas protegens* CHA0, plays a significant role in inter-bacterial competition and pest suppression (Marchi et al., 2013; Vacheron et al., 2019; Boak et al., 2022). Moreover, T6SS has been shown to contribute to bacterial killing and colonization in the rhizosphere, as demonstrated by the study on *Pseudomonas ogarae* F113 (Durán et al., 2021). It enables *P. putida* to secrete toxic metabolites that specifically target phytopathogens, providing protection to *Nicotiana benthamiana* plants against the pathogen *Xanthomonas campestris* (Bernal et al., 2017). Considering all these factors, research on *Pseudomonas* species, their genomic potential, and their modes of action in soils suppressive to *F. graminearum* diseases holds great importance. *Pseudomonas* may provide insights into the functioning of suppressive soils and offer potential solutions for combating the mycotoxicogenic *F. graminearum*.

GENERAL HYPOTHESES

Suppressive soils have already been documented in numerous parts of the world (Vasudeva & Roy, 1950; Alabouvette, 1986; Cha et al., 2016; Ossowicki et al., 2020) and it was observed that soil suppressiveness is influenced by the addition of organic matter, by positively affecting soil microbial diversity (Mousa & Raizada, 2016; Jayaraman et al., 2021). In a quest for suppressive soils, it can be assumed that it is worth investigating fungistatic soils, as: (i) fungistasis acts by inhibiting fungal propagules, therefore leaving less inoculum for the subsequent plant infection (Garbeva et al., 2011), and (ii) the possibility that fungistatic soils may also be disease-suppressive has already been mentioned in previous work (Lockwood, 1977; Garbeva et al., 2011; Milinković et al., 2019). On this basis, the general hypothesis of this project is that suppressive soils may be found worldwide and that soils with manure amendments are more likely to display fungistasis and even disease suppressivenes. This general hypothesis was subdivided into three specific hypotheses:

First hypothesis is that *Fusarium*-suppressive soils, which occur widely (Kyselková & Moënne-Loccoz, 2012; Cha et al., 2016), can be identified by screening of soils with limited disease problems (based on farmers' observations) or that have undergone organic matter-based management aiming to enhance microbial diversity.

Second hypothesis is that soil suppressiveness is driven by biotic factors and fungistatic (and suppressive) soils represent a reservoir of promising antagonists against soil-borne pathogen *F. graminearum*.

Third hypothesis is that genomic and functional analysis of fluorescent *Pseudomonas* isolates from suppressive vs. non-suppressive soils can be useful to explore soil suppressiveness mechanisms.

OBJECTIVES

The general objective of this project was to gain a better understanding of fungistasis and suppressiveness phenomena, and to assess their usefulness as sources of bacteria with biocontrol potential. To this end, we focused on mycotoxicogenic pathogen *F. graminearum*, as soils suppressive to diseases caused by different *Fusarium* species have been documented in different geographic regions, and because this pathogen can be influenced by fungistasis (Legrand et al., 2019).

This work was carried out in Serbia, because (i) fungistasis and disease suppressiveness *per se* have received no attention so far, even though plant-beneficial microorganisms have been extensively studied (Milinković et al., 2019; Karličić et al., 2020; Kerečki et al., 2022; Dragojević et al., 2023), including against *F. graminearum* (Karličić et al., 2022), (ii) it displays a combination of contrasted regions in terms of geography, soil type and farming management (Nejgebauer et al., 1971; Tanasijević et al., 1964), and (iii) there are soils with a history of manure amendment, while other soils nearby may not have been manured.

In this context, the first objective was to identify soils fungistatic and suppressive to *F. graminearum*, and investigate the relation between manure amendments and the occurrence of fungistasis/suppressiveness. To achieve this first objective, we chose 26 agricultural fields (with or without manure amendments), sampled in two contrasting regions in Serbia: (i) in the northern plains region of Serbia (i.e., Vojvodina), where the agriculture is more intensive, and soil is of type chernozem, and (ii) in the western/central hilly region of Serbia, where the agriculture is less intensive and soils are of type vertisols, eutric cambisols or pseudogleys. We tested the 26 soils for their fungistasis status, and afterwards, chosen fungistatic and non-fungistatic soils were chosen for *in planta* wheat phytoprotection assay with *F. graminearum*, and their fungal and prokaryotic rhizosphere diversity was compared.

The second objective aimed to assess the potential of *F. graminearum* fungistatic soils as a source of biocontrol agents. This involved isolation of bacteria of contrasted taxonomy, their characterization based on genomic and functional traits, and assessment of their wheat phytoprotective capacity against *F. graminearum*.

The third objective of this work was to identify the genomic and functional particularities of *Pseudomonas* bacteria in suppressive vs. non-suppressive soils. This was motivated by the fact that *Pseudomonas* may contribute to plant protection against *Fusarium* diseases and play a role in soil suppressiveness to these diseases, while biocontrol *Pseudomonas* have also been documented in non-suppressive soils. To achieve this comparison, the diversity of fluorescent *Pseudomonas* in the rhizosphere of wheat grown in suppressive and non-suppressive soils was analyzed using a metabarcoding approach targeting the *rpoD* gene of the *P. fluorescens* group. Subsequently, *Pseudomonas* were isolated from the rhizospheres of wheat plants grown in suppressive vs. non-suppressive soils and characterized based on genomic and functional traits.

STRUCTURE OF THE THESIS MANUSCRIPT

This manuscript is organized in 5 chapters. The first chapter serves as a comprehensive overview of the current state-of-the-art in the field. It begins by providing an introduction to the main concepts and types of soil suppressiveness, the significance of *Fusarium* pathogens, and an overview of the pathogen control methods that have been employed. Additionally, we delve into the topic of biocontrol agents targeting *Fusarium*, as well as a discussion of documented *Fusarium* suppressive soils, taking into account the influence of abiotic factors and farming practices on these soils. This review is titled "Microbial diversity in soils suppressive to *Fusarium* diseases" and has been accepted for publication in *Frontiers in Plant Science* in November, 2023.

The second chapter takes the form of a scientific article, entitled "Manure amendments and fungistasis, and relation with protection of wheat from *Fusarium graminearum*", and has been submitted to *Applied Soil Ecology* in September, 2023. In this chapter, we conduct tests on Serbian soils to assess their fungistasis (pathogen suppression) and suppressiveness (disease suppression) capabilities. Additionally, we explore the impact of manure amendments and soil physicochemical contents on fungistasis/suppressiveness. Then, we observe fungal and bacterial diversity and taxonomic composition in these soils.

The third chapter is also presented as a scientific article titled "Genomics of biocontrol bacteria from soils of contrasting suppressiveness status against *Fusarium graminearum*". In this chapter, we focus on bacterial isolates from fungistatic and non-fungistatic soils, and compare their genomes, specifically looking at the presence of genes related to biocontrol and plant-growth promotion. Here, we also provide a phenotypic characterization of selected bacteria and perform *in planta* phytoprotection assay using these bacteria and *F. graminearum*.

The fourth chapter is organized into 2 parts. The first part is titled "Two novel species isolated from wheat rhizospheres in Serbia: *Pseudomonas serbica* sp. nov. and *Pseudomonas serboccidentalis* sp. nov." and it describes the procedures followed to describe new species. The corresponding scientific article has been published in *Systematic and Applied Microbiology* in April, 2023. The second part is also presented as a scientific article titled "Fluorescent *Pseudomonas* from suppressive and non-suppressive soils share genomic and functional traits". In this part, we discuss fluorescent *Pseudomonas* isolated from suppressive and non-suppressive soils, as well as the comparison of their genomic potential, focusing on the presence of genes involved in biocontrol and plant-growth promotion.

Lastly, in the fifth chapter, we integrate and discuss the results from the second, third, and fourth chapters. Conclusions are drawn based on the findings, and we also address the limitations of the research and propose future perspectives for further studies.

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CHAPTER 1

Bibliographical synthesis: Microbial diversity in soils suppressive to *Fusarium* diseases

AVANT-PROPOS

In the natural environment, plants continuously interact with diverse and varied microorganisms. Over the past decade, the concept of the phytobiome has emerged, referring to the collective microbial communities within the plant ecosystem, including the plant itself, the soil, and associated organisms above- and below-ground (Hawkes & Connor, 2017; Leach et al., 2017). Essentially, the phytobiome corresponds to the plant holobiont and its surrounding environment. The phytobiome comprises the plant's microbiome, consisting of bacteria, fungi, archaea, viruses, and other microorganisms residing in various plant organs such as leaves, stems, roots, and flowers (Hacquard et al., 2015; Leach et al., 2017). It plays a crucial role in plant health, growth, development, and response to environmental stresses. It exerts influence on nutrient cycling, disease resistance, plant hormone regulation, and overall plant physiology (Vacheron et al., 2013). A comprehensive understanding of the composition and functions of the phytobiome is essential for achieving sustainable agriculture objectives, as it can facilitate the optimization of plant productivity, enhance disease management strategies, and reduce dependence on chemical inputs.

Despite their detrimental nature, phytopathogens also hold significant importance as components within the phytobiome. The *Fusarium* genus comprises several phytopathogenic species that cause significant damage to crops worldwide. These species produce mycotoxins and cause necrosis in economically important cereals, including wheat (Burgess & Bryden, 2012; Babadoost, 2018). Despite the availability of control methods such as chemical fungicides, resistant cultivars, and transgenic tools (Willocquet et al., 2021), *Fusarium* continues to inflict enormous crop losses in cereal-growing areas globally (Scott et al., 2021). In this context, suppressive soils represent a valuable model where diseases caused by phytopathogens can be suppressed. These soils harbor interactions among phytopathogens, beneficial soil microbiota and plant that result in improved plant health, even in the presence of the pathogen and under conditions suitable for disease development (Gómez Expósito et al., 2017). Soils suppressive to several phytopathogenic *Fusarium* species, i.e., *F. oxysporum*, *F. graminearum*, *F. culmorum* and *F. udum* have been documented worldwide, with several beneficial microbial taxa targeted as contributors to soil suppressiveness (Alabouvette, 1986; Cha et al., 2016; Ossowicki et al., 2020; Lv et al., 2023). The beneficial soil microbiota within suppressive soils protects plants and combats the phytopathogens using various modes of action, including the induction of systemic resistance, antagonism through the production of different compounds, competition for resources and parasitism (Nguvo & Gao, 2019). Moreover, specific agricultural practices can lead to the formation of suppressive soils, or impact soils that are already suppressive, by shaping and promoting the activity of the soil microbiome (Janvier et al., 2007; Campos et al., 2016).

Here, we will now present an overview of the current state of knowledge regarding suppressive soils in the context of diseases caused by *Fusarium* pathogens. This bibliographic synthesis serves several purposes. Firstly, it aims to provide a comprehensive summary of the current understanding of *Fusarium* pathogens, including their taxonomy, ecology, and mechanisms of plant infection, as well as the available control methods. Secondly, it seeks to summarize the existing knowledge on biocontrol agents targeting *Fusarium* and their underlying modes of action. Thirdly, it aims to consolidate the knowledge on soils that exhibit suppressiveness to *Fusarium* diseases, taking into account farming practices and ecological factors that may influence this suppressive behavior. Lastly, we will propose further research directions to address the existing knowledge gaps concerning suppressive soils in relation to *Fusarium* diseases.

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Microbial diversity in soils suppressive to *Fusarium* diseases

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ABSTRACT

Fusarium species are cosmopolitan soil phytopathogens from the division *Ascomycota*, which produce mycotoxins and cause significant economic losses of crop plants. However, soils suppressive to *Fusarium* diseases are known to occur, and recent knowledge on microbial diversity in these soils has shed new lights on phytoprotection effects. In this review, we synthesize current knowledge on soils suppressive to *Fusarium* diseases and the role of their rhizosphere microbiota in phytoprotection. Different approaches, such as the use of fungicides, resistant plant cultivars or post-harvest measures have been used to control *Fusarium* pathogens, but none of them is completely efficient or safe for the environment. Hence the importance of suppressive soils, in which disease does not develop significantly even though pathogenic *Fusarium* and susceptible host plant are present and weather conditions are suitable for disease. Soils suppressive to *Fusarium* diseases are documented in different regions of the world. They contain biocontrol microorganisms, which act by inducing plants' resistance to the pathogen, competing with or antagonizing the pathogen, or parasitizing the pathogen. In particular, some of the *Bacillus*, *Pseudomonas*, *Paenibacillus* and *Streptomyces* species are involved in plant protection from *Fusarium* diseases. Besides specific bacterial populations involved in disease suppression, next-generation sequencing and ecological networks have largely contributed to the understanding of microbial communities in soils suppressive or not to *Fusarium* diseases, revealing different microbial community patterns and differences for a notable number of taxa, according to the *Fusarium* pathosystem, the host plant and the origin of the soil. Agricultural practices can significantly influence soil suppressiveness to *Fusarium* diseases by influencing soil microbiota ecology. Research on microbial modes of action and diversity in suppressive soils should help guide the development of effective farming practices for *Fusarium* diseases management in sustainable agriculture.

Keywords: deoxynivalenol, nivalenol, zearalenone, Fusarium head blight, induced systemic resistance, lipopolysaccharides

INTRODUCTION

The fungal genus *Fusarium* encompasses several plant-pathogenic species, which are among the most destructive phytopathogens world-wide, causing diseases on many agricultural crops (Burgess and Bryden, 2012). They are ubiquitous in parts of the world where cereals and other crops are grown and they produce a wide variety of mycotoxins, which may be present in feed and food products (Moretti et al., 2018; Babadoost, 2018; Chen et al., 2019). Consumption of products that are contaminated with mycotoxins may cause acute or chronic effects in both animals and humans, and could result in immune-suppressive or carcinogenic effects (Jard et al., 2011). By producing mycotoxins and by inducing necrosis and wilting in plants, *Fusarium* fungi are causing huge economic losses of cereal crops throughout the world (Khan et al., 2017). Their broad distribution has been attributed to their ability to develop on different substrates and plant species, and to produce spores that enable efficient propagation (Desjardins, 2006; Arie, 2019). They are typical soil-borne microorganisms, routinely found in plant-associated fungal communities (Reyes Gaige et al., 2020).

Efficient management of plant diseases caused by *Fusarium* is important to limit crop losses and to reduce mycotoxin production in alimentary products (Babadoost, 2018). Because mycotoxin synthesis can occur not only after harvesting but also before, one of the best ways to reduce its presence in food and feed products is to prevent its formation in the crop (Jard et al., 2011). Over the years, different methods, such as the use of resistant cultivars and chemical fungicides, have been undertaken in order to control or prevent crop diseases (Willocquet et al., 2021). In spite of that, *Fusarium* continues to cause huge crop losses, up to 70% in South America, 54% in the United States and 50% in Europe in the case of Fusarium head blight (FHB) disease of wheat (Scott et al., 2021).

Alternative control methods, based on plant-protection effects of beneficial microorganisms, have also been investigated (Janvier et al., 2007; Nguyen et al., 2018). Farming practices greatly influence these effects by shaping the rhizosphere microbial community (Campos et al., 2016), stimulating the activity of beneficial rhizosphere microorganisms and restricting the activity of soil-borne *Fusarium* pathogens (Janvier et al., 2007). Indeed, crop rotation, tillage and addition of organic amendments may provide some control of soil-borne pathogens, through different microbial direct and indirect mechanisms (Janvier et al., 2007). The effect of plant-protecting soil microbiota on plant health is of particular interest in the case of disease-suppressive soils, which were defined by Baker and Cook (1974) as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil". Suppressive soils represent a reservoir of beneficial microorganisms, which may confer effective plant protection against various soil-borne phytopathogens (Gómez Expósito et al., 2017). This biocontrol potential of suppressive soils is of great importance when considering phytopathogens like *Fusarium*, which are causing increasing damage to crops in the on-going climate change context (Babadoost, 2018). Insight into the time and space microbial dynamics of soils suppressive to *Fusarium* diseases, together with the understanding of microbial modes of action and agricultural practices applied, is needed in order to develop safe, effective, and stable tools for disease management (Gómez Expósito et al., 2017).

This review deals with recent knowledge on soils suppressive to *Fusarium* diseases, which sheds new lights on molecular and ecological mechanisms underpinning phytoprotection effects and highlights the importance of microbial diversity in the functioning of these suppressive soils. To this end, we summarize current knowledge on *Fusarium* taxonomy and ecology, their mechanisms of plant infection, and the chemical, genetic and post-harvest control methods available. In addition, we review our understanding of biocontrol agents against *Fusarium* and their modes of action. Finally, we focus on soils

suppressive to *Fusarium* diseases and the importance of farming and environmental factors modulating suppressiveness, with an emphasis on the particularities of the different *Fusarium* pathosystems.

SOIL MICROBIAL COMMUNITIES AND OCCURRENCE OF SOIL SUPPRESSIVENESS: MAIN CONCEPTS AND TYPES OF SOIL SUPPRESSIVENESS

Soil represents the richest known reservoir of microbial biodiversity (Curtis et al., 2002; Wang et al., 2016) and displays several compartments, i.e., the bulk soil containing microorganisms that are not affected by the roots, the rhizosphere where soil microorganisms are under the influence of roots (and roots exudates) and the rhizoplane with root-adhering microorganisms (Sánchez-Cañizares et al., 2017). The rhizosphere and rhizoplane harbor an abundant community of bacteria, archaea, oomycetes and fungi, whose individual members can have beneficial, deleterious or neutral effects on the plant. The collective genome of this microbial community is larger than that of the plant itself, and is often referred to as the plant's second genome (Berendsen et al., 2012). Thus, this alliance of the plant and its associated microorganisms represents a holobiont, which has interdependent, fine-tuned and complex functioning (Berendsen et al., 2012; Vandenkoornhuyse et al., 2015; Sánchez-Cañizares et al., 2017). In this system, a plant is a key player, as nearly 40% of all photosynthates are released directly by roots into the rhizosphere, serving as a fuel for microbial communities, thus recruiting and shaping this microbiome (Berendsen et al., 2012; Tkacz and Poole, 2015). These photosynthates are conditioned by the plant genotype, developmental stage, metabolism, immune system and its ability to exudate (Sánchez-Cañizares et al., 2017). By the selection of its rhizosphere microbiome, plants contribute to the suppressiveness of soils, where diseases caused by pathogens may be controlled (Tkacz et al., 2015).

Soils that are suppressive to soil-borne diseases have been known for more than 70 years (Vasudeva and Roy, 1950), and disease suppression is associated primarily with the activity of beneficial microorganisms (Schlatter et al., 2017). These microorganisms interact with phytopathogens, thus affecting their survival, development or infection of the plant (Weller et al., 2002; Raaijmakers et al., 2009). Two types of soil suppressiveness have been described, i.e., general (microbial community-based) suppressiveness and specific (microbial population-based) suppressiveness (Schlatter et al., 2017). General suppressiveness is dependent on the entire soil microbial biomass, which causes pathogen inhibition through various mechanisms, especially competition, and it cannot be transferred experimentally between the soils (Weller et al., 2002). Hence, all soils may present some level of general suppressiveness to soil-borne diseases, and this level depends on soil type, agricultural practices and total microbial activity (Janvier et al., 2007; Raaijmakers et al., 2009). Besides general suppressiveness, there is specific suppressiveness to certain diseases, which relies on the activity of a few plant-protecting microbial groups (Weller et al., 2007; Almario et al., 2014; Mousa and Raizada, 2016). Specific suppressiveness may be conferred to non-suppressive soils (i.e., conducive soils) by inoculating them with 0.1% - 10% of suppressive soil (Garbeva et al., 2004; Raaijmakers et al., 2009). Although abiotic factors, such as soil physicochemical properties, may contribute to the control of a given pathogen, specific suppressiveness is essentially a phenomenon mediated by beneficial soil microorganisms, since sterilization processes convert suppressive into conducive soils (Garbeva et al., 2004). It is expected that specific suppressiveness entails the contribution of a few plant-protecting microbial groups (Weller et al., 2007), but microbial community comparison of suppressive vs. conducive soils may evidence significant differences for a large number of taxa (Kyselková et al., 2009; Legrand et al., 2019; Ossowicki et al., 2020; Yuan et al., 2020; Lv et al., 2023).

FUSARIUM PHYTOPATHOGENS AND PLANT DISEASES

Fusarium ecology

Fusarium species occur in soils, but they can also grow in and on living and dead plants and animals, with the ability to live as parasites or saprophytes (Smith, 2007). Some can also be found in caves (Bastian et al., 2010) or in man-made water systems (Sautour et al., 2012). *Fusarium* species are mostly known as phytopathogens, but some of them have been evidenced as contaminants in industrial processes, in indoor environments, or in pharmaceutical and food products (Abdel-Azeem et al., 2019).

The saprophytic potential of *Fusarium* species enables them to survive the winter in the crop debris, in the form of mycelium or spores that serve as plant-infecting propagules in the spring (Figure 1A) (Leslie and Summerell, 2006). *Fusarium* species vary in reproduction strategies, and they produce sexual spores (ascospores) as well as three types of asexual spores, i.e., (i) microconidia, which are typically produced under all environmental conditions, (ii) macroconidia, which are often found on the surface of diseased plants, and (iii) chlamydospores (survival structures), which are thick walled and produced from macroconidia or older mycelium (Ajmal et al., 2023). More than 80% of *Fusarium* species propagate using asexual spores, but not all of them produce all three types of spores, while sexual reproduction can involve self-fertility or out-crossing (Rana et al., 2017). Additionally, some species produce sclerotia, which promote survival in soil (Leslie and Summerell, 2006).

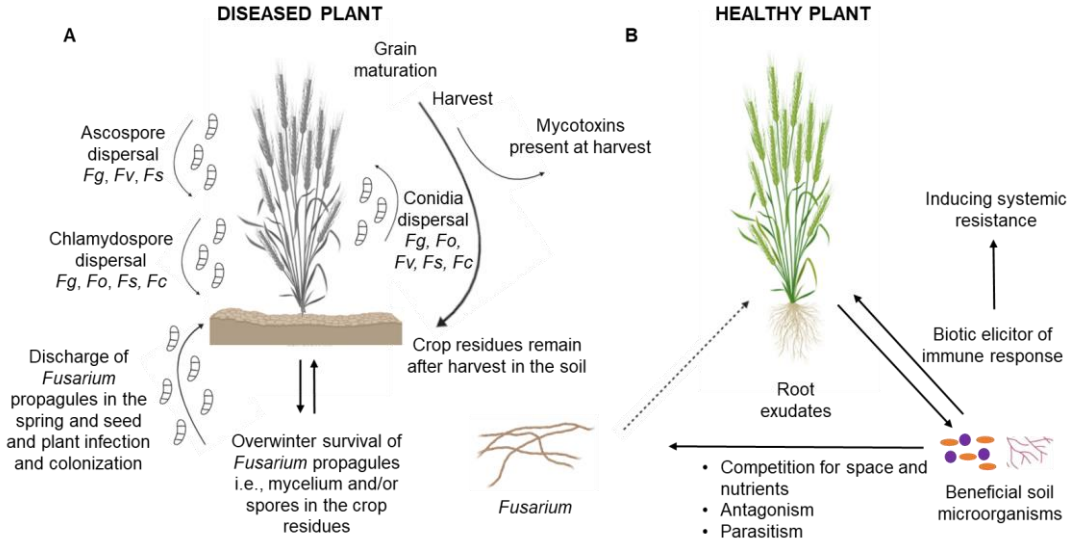

Figure 1. Interactions of *Fusarium* species with plant and other microbiota members. (A) Life cycle of *Fusarium* species and their mechanism of plant infection by producing three types of spores: ascospores, conidia and chlamydospores. Abbreviations: *Fg*, *Fusarium graminearum*; *Fo*, *Fusarium oxysporum*; *Fs*, *Fusarium solani*; *Fc*, *Fusarium culmorum*; *Fv*, *Fusarium verticillioides*. (B) Dynamic interactions between beneficial soil microorganisms, plant and phytopathogenic *Fusarium* species.

Fusarium shows climatic preferences, as *F. oxysporum*, *F. solani*, *F. verticillioides* (formerly *F. moniliforme*) and *F. graminearum* are found worldwide, *F. culmorum* in temperate regions, whereas some species occur in tropical or cool regions (Babadoost, 2018). The growth of each *Fusarium* species is largely determined by abiotic environmental conditions, notably temperature and humidity (Table S1) (Xu, 2003). However, other environmental factors, such as soil characteristics, cropping systems, agricultural practices and other human activities may influence the diversity of *Fusarium* in soils (Abdel-Azeem et al., 2019).

Taxonomy of *Fusarium*

The *Fusarium* genus exhibits high level of variability in terms of morphological, physiological and ecological properties, which represents a difficulty in establishing a consistent taxonomy of these species (Burgess et al., 1996). An additional difficulty for classification is the existence of both asexual (anamorph) and sexual (teleomorph) phases in their life cycle (Summerell, 2019). Based on the most widely used classification, the anamorph state of the genus *Fusarium* is classified in the family *Nectriaceae*, order *Hypocreales* and division *Ascomycota* (Crous et al., 2021). Several teleomorphs have been related to *Fusarium* species, but not all *Fusarium* species have a known sexual state in their life cycle (Munkvold, 2017). Most of these teleomorphs are in the genus *Gibberella*, including the economically important pathogens, such as *G. zeae* (anamorph *F. graminearum*) and *G. moniliformis* (anamorph *F. verticillioides*) (Keszthelyi et al., 2007). Other *Fusarium* teleomorphs are members of the genera *Albonectria*, *Neocosmospora* or *Haematonectria*. Teleomorphs are usually not observed in the field, but rather under lab conditions. The dual anamorph-teleomorph nomenclature for fungi has now been abolished, and the name *Fusarium* has been retained for these fungi (Geiser et al., 2013).

The genus *Fusarium* is currently composed of 23 species complexes and at least 69 well-individualized species. *Fusarium* species complexes are groups of closely-related species with the same morphology, which are strongly supported from a phylogenetic perspective (O'Donnell et al., 2013; Summerell, 2019), as shown in Figure 2. Within a given *Fusarium* species, certain strains may be pathogenic while others are not (Fuchs et al., 1997). However, most phytopathogenic species belong to the *F. fujikuroi*, *F. sambucinum*, *F. oxysporum* or *F. solani* species complexes (O'Donnell et al., 2013). Furthermore, *Fusarium* species capable of infecting a wide range of plants are classified into different *formae speciales,* based on the host plant they can infect (Coleman, 2015; Edel-Hermann and Lecomte, 2019). Currently, there are 106 well-described *F. oxysporum formae speciales* (Edel-Hermann and Lecomte, 2019) and 12 well-described *F. solani formae speciales* (Šišić et al., 2018).

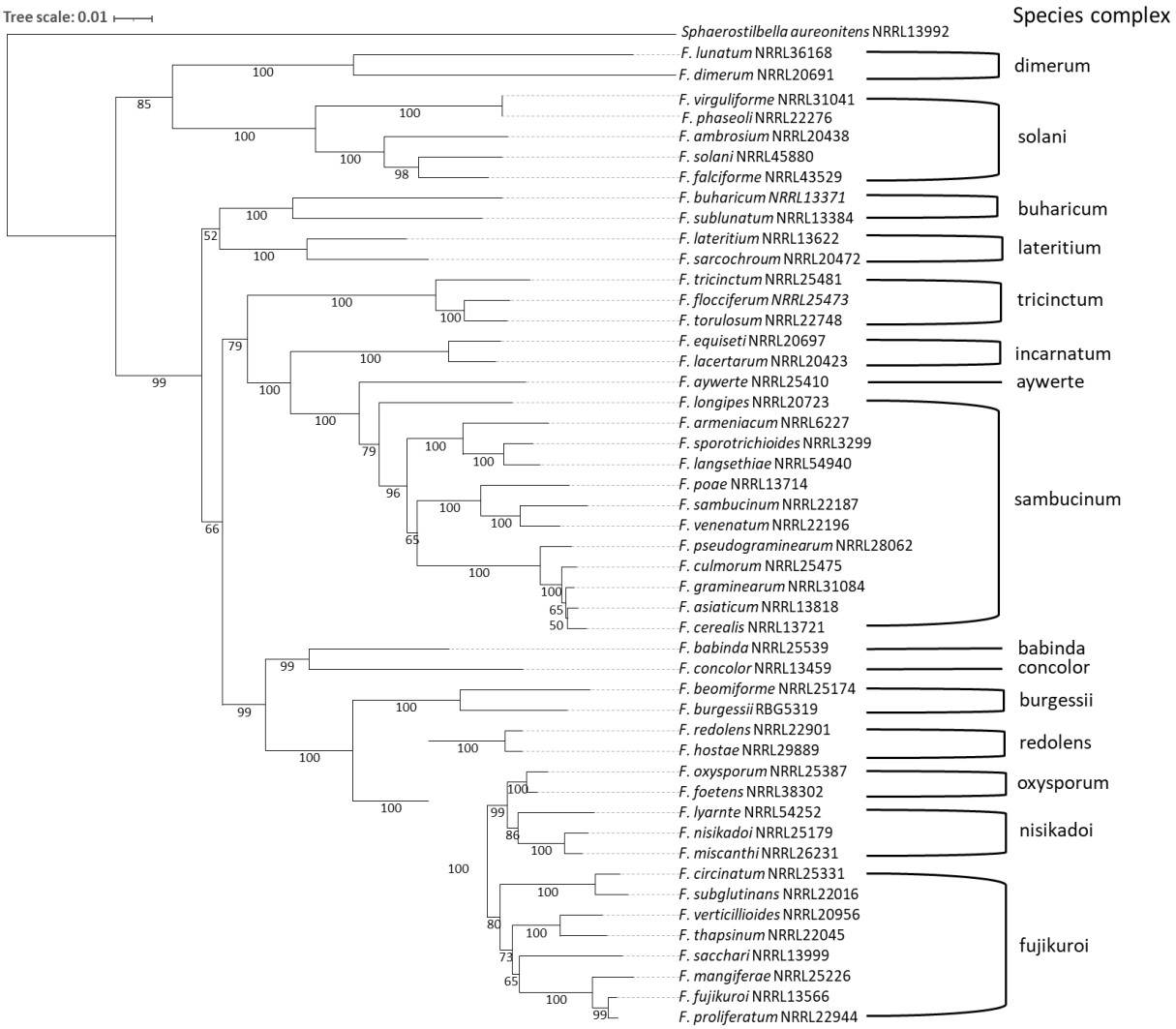

Figure 2. Phylogenetic relationship between different *Fusarium* species within different species complexes. The distance-method tree (1000 bootstrap replicates) was inferred from the *rpb1* (RNA Polymerase 1) data set, using the SeaView multiplatform (Gouy et al., 2010). The tree was visualized using iTol (Letunic and Bork, 2021). *Sphaerostilbella aureonitens* NRRL 13992 was used as an outgroup.

Over the past 100 years, the taxonomy of *Fusarium* has undergone many changes, but most classification procedures have been based on the size and shape of the macroconidia, the presence or absence of microconidia and chlamydospores, and the structure of the conidiophores (Ristić, 2012). Identification of *Fusarium* species based on morphological characteristics also included observations of colony pigmentation and type of aerial mycelium (Crous et al., 2021). The standard method now used to identify *Fusarium* isolates to a species level is to sequence one (or more) of the following genes: translocation elongation factor-1α (*tef-1α*), RNA polymerase 1 and 2 (*rpb1* and *rpb2*), β-tubulin (*tub*), histone (*his*), ATP citrate lyase (*acl1*) or calmodulin (*CaM*) (Herron et al., 2015; Summerell, 2019; Crous et al., 2021). The *tef-1α* gene is a first-choice marker as it has good resolution power for the majority of *Fusarium* species, while sequencing the gene *rpb2* allows differentiation of close species. The other genetic markers mentioned have variable resolution power and are often used together with *tef-1α* or *rpb2* (Crous et al., 2021). The internal transcribed spacer regions of the ribosomal gene (ITS), which are common barcodes to identify fungi, are not recommended for *Fusarium* identification, as they are not sufficiently informative for a significant number of *Fusarium* species (Summerell, 2019).

Mechanisms of *Fusarium* infection, symptoms and etiology

Before infecting the host plant tissues, soil-borne pathogens may grow in the rhizosphere or on the host as saprophytes, managing to escape the rhizosphere battlefield (Raaijmakers et al., 2009). The outcome is directly influenced by host and microbial defense mechanism, at the level of the holobiont (Berendsen et al., 2012; Vandenkoornhuyse et al., 2015). During their life cycle, plants are exposed to numerous phytopathogens, and they have developed different adaptive strategies. Upon pathogen attack, both composition and quantity of root metabolites may change (Rolfe et al., 2019), which can be useful for direct defense against the pathogens (Rizaludin et al., 2021), for signaling the impending threat to the neighboring plants (Pélissier et al., 2021), or for recruiting beneficial microorganisms with biocontrol capabilities. The latter phenomenon is referred to as the 'cry for help' strategy (Rizaludin et al., 2021).

If the pathogen manages to escape from the rhizosphere battlefield, the infection cycle can proceed. Plant infection by *Fusarium* occurs in a few successive stages (Figure 1A), which differs according to *Fusarium* species. Seeds infected with *Fusarium* in the previous season can also serve as disease initiators (Jiménez-Díaz et al., 2015). *F. graminearum* grows saprophytically on crop debris, which is the overwintering reservoir of the pathogen (Brown et al., 2010). The fungus may infect roots and cause damage to the collar (Ares et al., 2004). During the crop anthesis and under warm and humid weather conditions, asexual conidia, sexual ascospores or chlamydospores are dispersed by rain or wind and reach the outer anthers and outer glumes of the plant. After spore germination, hyphae penetrate the host plant through the cracked anthers, followed by inter- and intracellular mycelial growth, resulting in damage to host tissues and especially head blight disease (Brown et al., 2010). Unlike *F. graminearum*, *F. culmorum* produces only asexual conidia and chlamydospores, which are also dispersed by rain and wind, reaching plant heads and infecting the ears during the anthesis. Subsequently, conidia germinate on lemma and palea, followed by inter- and intracellular mycelial growth (Wagacha and Muthomi, 2007). In contrast, the infection cycle of *F. oxysporum* begins when mycelia, germinating asexual conidia or chlamydspores enter the healthy plant through the root tip, lateral roots or root wounds. The fungus progresses intracellularly, entering the xylem sap flow and being transported to the aerial parts of the plant where it forms infection structures. The infection structures that form close the vascular vessels, disrupt nutrient translocation, leading to stomatal closure, leaf wilting and plant death (Banerjee and Mittra, 2018). In the case of *F. verticillioides*, infection starts when mycelia, asexual conidia or sexual ascospores are carried inside the seed or on the seed surface and later develop inside the growing plant, moving from the roots up to the maize kernels (Oren et al., 2003). Sometimes, the fungus colonizes and grows along the veins of the plant root, while sometimes it manages to penetrate the plant cells and form internal hyphae, therefore causing damage (Lei et al., 2011). Finally, for *F. solani*, the attachment of mycelia, asexual conidia, sexual ascospores or chlamydospores to the susceptible host is the first step in disease development, after which the fungus enters the host through stomata or the epidermis. Following penetration, *F. solani* is able to spread through the xylem, ultimately causing wilting of the host plant (Coleman, 2015).

It is reported that mycotoxins play a key role in pathogenesis and that the aggressiveness of *Fusarium* depends on its toxin-producing capacity (Mesterházy, 2002). There are several mycotoxins produced by *Fusarium* species, such as the trichothecenes deoxynivalenol (DON) and nivalenol (NIV), zearalenone (ZEA) and fusaric acid (Wagacha and Muthomi, 2007), and the biosynthesis of these toxins is encoded by the *tri*, *pks* and *fus* genes, respectively (Dhanti et al., 2017). However, not every species has the ability of producing all of the abovementioned mycotoxins. For example, DON and NIV are commonly produced by *F. graminearum* and *F. culmorum,* while ZEA and fusaric acid are often produced by *F. graminearum*, *F. culmorum* and *F. verticillioides* (Nešić et al., 2014). DON production by *F.*

graminearum is reported to be essential for disease development in wheat spikes (Cuzick et al., 2008). Spikes treated with DON or NIV led to yield losses even in the absence of the pathogen, indicating a strong negative effect of these trichothecenes on wheat growth (Ittu et al., 1995). In addition to DON, fusaric acid is also a virulence factor involved in programmed cell death (López-Díaz et al., 2018). It was shown that alkaline pH and low nitrogen and iron availabilities led to increased fusaric acid production in *F. oxysporum* (Palmieri et al., 2023). Besides mycotoxins, there are other metabolites produced by *Fusarium* species that play a role in disease pathogenesis. Deletion of the *F. graminearum* gene cluster responsible for the synthesis of fusaoctaxin A abolished the fungal ability to colonize wheat coleoptiles (Jia et al., 2019). Extracellular lipases secreted by *F. graminearum* affected the plant's defense responses by inhibiting callose synthase activity (Blümke et al., 2014).

Diseases caused by *Fusarium* species include blights, wilts and rots of various crops in natural environments and in agroecosystems (Nelson et al., 1994; Ma et al., 2013). FHB or 'scab' is a disease caused primarily by the *F. graminearum* species complex. It is the fourth-ranked fungal phytopathogen in term of economic importance (Dean et al., 2012; Legrand et al., 2017), causing yield losses of 20% to 70% (Bai and Shaner, 1994). *F. graminearum* is responsible for kernel damage and mycotoxin production (Ma et al., 2013) in cereals like wheat, barley, rice and oats (Goswami and Kistler, 2004). Typical symptoms of FHB begin soon after flowering, as diseased spikelets gradually bleach, leading to bleaching of the entire head. After this stage, black spherical structures called perithecia may appear on the surface of diseased spikelets. Later, as the disease becomes more severe, the fungus begins to attack the kernels inside the head, causing them to wrinkle and shrink (Schmale and Bergstrom, 2003). FHB can also be caused by *F. culmorum*, which is dominant in cooler regions of Europe (Wagacha and Muthomi, 2007). Vascular wilt is responsible for severe losses in crops such as melon, tomato, cotton, bean and banana. It is caused by *F. oxysporum*, the fifth most economically important fungal phytopathogen (Michielse and Rep, 2009; Dean et al., 2012; Husaini et al., 2018). Symptoms of vascular wilt are first observed on the older leaves, as they begin to droop, followed by defoliation and yellowing of the younger leaves and eventually, plant death (Britannica, 2017). Root, stem and foot rots of various non-grain host plants are often caused by *F. solani*, and the disease symptoms depend on the host plant and the particular *forma specialis* (Voigt, 2002; Coleman, 2016). However, typical symptoms of root, stem and foot rots include brown lesions on the affected plant organs. *F. verticillioides* causes ear and stalk rot in hosts such as maize, sorghum and rice (Murillo-Williams and Munkvold, 2008; Dastjerdi and Karlovsky, 2015), whereas *F. graminearum* is responsible for causing *Fusarium* ear and stalk rot in maize (Goswami and Kistler, 2004). *Fusarium* ear rot is characterized by discoloration of single or multiple kernels in different areas of the ear, while early signs of stalk rot include lodging and discoloration of the stem.

CHEMICAL, GENETIC AND POST-HARVEST CONTROL METHODS

Chemical fungicides

Several studies have been conducted to evaluate the efficacy of fungicides in reducing *Fusarium* diseases and mycotoxin levels in harvested cereals. The demethylation inhibitor class of fungicides, consisting of triazoles and imidazoles, is one of the most widely used group of fungicides to suppress *Fusarium* growth. Fungicides in this class work by inhibiting the demethylation step in sterol biosynthesis (Nel et al., 2007). Triazole fungicides are widely used to control FHB caused by *F. graminearum* (Li and Liu, 2022). Prothioconazole, a 1,2,4-triazole fungicide, applied before wheat head emergence, can reduce FHB by up to 97% and DON production by 83% (Edwards and Godley, 2010). In contrast, Li and Liu (2022) found that prothioconazole enantiomers increased DON production. Metconazole, another triazole

fungicide, and its stereoisomers (1S, 5R) are effective in controlling *F. verticillioides*, and they can be used at lower dosages with less environmental impact, because they have higher bioactivity than other metconazole stereoisomers (Li et al., 2022). It is an important issue, as long-term use of fungicides leads to residual contamination of soils and potentially harmful effects on end users, both animals and humans (Zhang et al., 2020). The imidazole fungicide prochloraz and the triazole fungicide propiconazole significantly inhibited the development of *F. oxysporum* when applied as a root-dip treatment to the banana cultivar Chinese Cavendish (Nel et al., 2007). However, contrary to *F. oxysporum*, *F. fujikuroi* has developed strong resistance to prochloraz (Gao et al., 2022). Carbendazim and other benzimidazole fungicides used to be very effective against *F. fujikuroi*, *F. proliferatum*, and *F. verticillioides*, but over time, fungicide-resistant subpopulations of this pathogen have emerged, thus leading to control failures (Chen et al., 2014).

Genetic resistance

In the case of FHB in wheat, caused by *F. graminearum*, a few resistant cultivars have been described. They confer resistance either during the initial infection, during pathogen dissemination within the spike or during the mycotoxin production (Chen et al., 2019). These different types of resistance to FHB are quantifiable, and they are controlled by various quantitative trait loci (QTLs) i.e., more than 550 QTLs located throughout the whole wheat genome (Venske et al., 2019; Fabre et al., 2020). For example, the resistance of the Chinese wheat cultivar Sumai-3 is controlled by the Fhb1 QTL on chromosome 3BS, which is the most stable and efficient resistance locus, allowing a relatively high level of resistance to *Fusarium* spread within the spike (Chen et al., 2019). Although a large number of QTLs have been documented, only few of them have been successfully used to develop resistant varieties (Venske et al., 2019).

Besides resistance traits, certain plant varieties possess susceptibility factors, coded by susceptibility (S) genes, that promote pathogen proliferation and disease development (Vogel et al., 2002; Chetouhi et al., 2015; Fabre et al., 2020). In the susceptible wheat cultivar Récital, grain infection by *F. graminearum* does not significantly affect grain development, but affects primary metabolism by altering starch biosynthesis and storage proteins. Although little studied compared to resistant traits, susceptibility factors may be crucial in determining the outcome of pathogen attack, opening up the possibility of developing FHB control strategies based on loss of susceptibility genes (Fabre et al., 2020).

Transgenic tools have also been proposed to control *Fusarium* diseases, in particular host-induced gene silencing (HIGS). This approach is based on engineering plants to produce interfering RNAs, that are mobile and able to enter fungal cells. Once inside, they trigger the degradation of transcripts of essential genes, such as chitin synthetase and DON-encoding *Tri5* genes. Thus, HIGS has the potential to reduce *Fusarium*-caused diseases under field conditions and to minimize mycotoxin contamination of crops (Cheng et al., 2015).

Post-harvest control

Although the best way of coping with mycotoxins would be to prevent their formation in crops, another possibility is to develop post-harvest processes to detoxify already-contaminated feed and food products. The most promising strategies include (i) adsorption, which involves the use of adsorbents that bind mycotoxins in the gastrointestinal system and reduce their absorption and toxicity, (ii) microbial degradation, which involves the removal of the mycotoxins, and (iii) microbial transformation of mycotoxins into less toxic compounds (Awad et al., 2010; Vanhoutte et al., 2016). Hsu et al. (2018) suggested that *Bacillus licheniformis* CK1 could be formulated as a feed additive, due to its ability to adsorb ZEA, and

form a ZEA-CK1 complex, which can then be eliminated through the animal's gastrointestinal system. The fungus *Clonostachys rosea* has been shown to degrade ZEA using a zearalenone lactonohydrolase (Kosawang et al., 2014). A soil bacterium of the *Agrobacterium-Rhizobium* genus complex converts DON to the less toxic 3-keto DON (Shima et al., 1997). *Burkholderia ambifaria* has the ability to degrade fusaric acid, by using it as the sole source of carbon and nitrogen (Simonetti et al., 2018). However, the applicability of this strategy is not clear in the case of multiple mycotoxin contamination of food and feed, and not all transformations lead to less toxic or non-toxic products (Vanhoutte et al., 2016).

BIOCONTROL AGENTS AGAINST *FUSARIUM* AND THEIR MODES OF ACTION

Plant-beneficial microorganisms present in the rhizosphere may protect plants from *Fusarium* pathogens, through different modes of action including (i) induction of resistance in the plant, (ii) competition with the pathogens for space and nutrients, (iii) antagonism based on the production of different metabolites or (iv) parasitism (Figure 1B) (Nguvo and Gao, 2019; Morimura et al., 2020). Some of them are also able to inhibit mycotoxin synthesis or to enhance their detoxification (Legrand et al., 2017; Morimura et al., 2020). Certain biocontrol microorganisms have multiple modes of action, which may be expressed simultaneously or sequentially (Legrand et al., 2017).

Induced systemic resistance

Induced Systemic Resistance (ISR) is the phenomenon whereby a plant, once appropriately stimulated by biological or chemical inducers, exhibits enhanced resistance when challenged by a pathogen (Walters et al., 2013). ISR involves (i) the plant perception of inducing signals, (ii) signal transduction by plant tissues, and (iii) expression of plant mechanisms inhibiting penetration of the pathogen into the host tissues (Magotra et al., 2016). A wide variety of microorganisms, including the bacteria *Pseudomonas, Bacillus, Streptomyces* and the fungi *Trichoderma* and non-pathogenic *F. oxysporum* can induce ISR (Fuchs et al., 1997; Choudhary et al., 2007; Zhao et al., 2014; Galletti et al., 2020) in plants against *Fusarium* (Table 1). ISR in the plant-*Fusarium* system is based on microbial induction of the activity of various defense-related enzymes in plants, such as chitinase (Amer et al., 2014), lipoxygenase (Aydi Ben Abdallah et al., 2017), polyphenol oxidase (Akram et al., 2013), peroxidase, phenylalanine ammonia-lyase (Zhao et al., 2012), β-1,3-glucanase, catalase (Sundaramoorthy et al., 2012), and also the accumulation of phytoalexins, defense metabolites against fungi (Kuć, 1995). Cyclic lipopeptide antibiotics, e.g., fusaricidin (Li and Chen, 2019) and external cell components, e.g., lipopolysaccharides (Leeman et al., 1995) can also trigger ISR. Some biocontrol agents can lead to ISR in different plant species, while other biocontrol agents show plant species specificity, suggesting specific recognition between microorganisms and receptors on the root surface (Choudhary, 2007).

Table 1. Biocontrol agents, plant-*Fusarium* systems and ISR mechanisms.

Biocontrol agent	Plant	Pathogen	Mechanism	Reference
Bacillus amyloliq uefaciens	Tomato	*F. oxysporum*	Induction of genes coding for lipoxygenase or pathogenesis-related (PR) proteins, i.e., acidic protein PR-1 and PR-3 chitinases	Aydi Ben Abdallah et al., 2017
Bacillus thuringiensis	Tomato	*F. oxysporum*	Increase in polyphenol oxidase, phenyl ammonia lyase and peroxidase in plant	Akram et al., 2013
Bacillus megaterium	Tomato	*F. oxysporum*	Induction of chitinase, β-1,3-glucanase, peroxidase and polyphenol oxidase activities in plant	Amer et al., 2014
Bacillus subtilis	Tomato	*F. oxysporum*	Increased activities of phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase enzymes in plant	Akram et al., 2015
Bacillus subtilis and *Pseudomonas protegens* (in combination and alone)	Chilli	*F. solani*	Increased activities of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, β-1,3-glucanase, chitinase enzymes and phenol compounds involved in the synthesis of phytoalexins	Sundaramoorthy et al., 2012
Bacillus sp., *Brevibacillus brevis* and *Mesorhizobium ciceri* (in combination)	Chickpea	*F. oxysporum*	Increase in peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, phenols and total proteins in plants	Kumari and Khanna, 2019
Brevibacillus parabrevis	Cumin	*F. oxysporum*	Increase in peroxidase and polyphenol oxidase in plants	Abo-Elyousr et al., 2022
Burkholderia gladioli	Saffron	*F. oxysporum*	Increased levels of endogenous jasmonic acid (JA) and expression of JA-regulated and plant defense genes	Ahmad et al., 2022
Pseudomonas aeruginosa	Tomato	*F. oxysporum*	Bacterial production of 3-hydroxy-5-methoxy benzene methanol	Fatima and Anjum, 2017
Pseudomonas simiae	Tomato	*F. oxysporum*	Bacterial production of lipopolysaccharides	Duijff et al., 1997

Pseudomonas defensor	Radish	*F. oxysporum*	Bacterial production of lipopolysaccharides	Leeman et al., 1995
Paenibacillus polymyxa	Cucumber	*F. oxysporum*	Bacterial production of fusaricidin, which induces ISR via salicylic acid	Li and Chen, 2019
Pseudomonas fluorescens	Barley	*F. culmorum*	Changed transcript levels of lipid transfer proteins and protease inhibitors	Petti et al., 2010
Streptomyces enis socaesilis	Tomato	*F. oxysporum*	Increased catalase activity in plant	Abbasi et al., 2019
Streptomyces roc hei	Tomato	*F. oxysporum*	Increased catalase and peroxidase activity in plant	Abbasi et al., 2019
Streptomyces bikiniensis	Cucumber	*F. oxysporum*	Increased activities of peroxidase, phenylalanine ammonia-lyase, and β-1,3-glucanase in plant	Zhao et al., 2012
Trichoderma gamsii	Maize	*F. verticillioides*	Enhanced transcript levels of ISR marker genes	Galletti et al., 2020
Trichoderma longibrachiatum	Onion	*F. oxysporum*	Accumulation of 25 stress-response metabolites	Abdelrahman et al., 2016
Non-pathogenic *Fusarium oxysporum*	Tomato	*F. oxysporum*	Increased activities of chitinase, β-1,3-glucanase and β-1,4-glucosidase	Fuchs et al., 1997

Bacillus amyloliquefaciens subsp. *plantarum* strain SV65 was assessed on tomato plants infected or not with *F. oxysporum* f. sp. *lycopersici* (FOL). The expression of genes coding for lipoxygenase or pathogenesis-related (PR) proteins, i.e., acidic protein PR-1 and PR-3 chitinases was induced by *B. amyloliquefaciens* subsp. *plantarum* SV65 in both FOL-inoculated and uninoculated plants, suggesting its ability to induce ISR (Aydi Ben Abdallah et al., 2017). Inoculation of chilli plants with *Bacillus subtilis* EPCO16 and EPC5, and *Pseudomonas protegens* Pf1, separately or in combination, induced ISR, with enhanced phytoalexin activities, and protected plants against *F. solani* (Sundaramoorthy et al., 2012). Inoculation of chickpea plants with a combination of *Bacillus* sp., *Brevibacillus brevis* and *Mesorhizobium ciceri* led to the accumulation of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenols in plants as well as resistance to *F. oxysporum* (Kumari and Khanna, 2019). *Paenibacillus polymyxa* WLY78 controls Fusarium wilt, caused by *F. oxysporum* f. sp. *cucumerinum*, through the production of fusaricidin, which can induce ISR in cucumber via the salicylic acid pathway (Li and Chen, 2019). Tomato showed increased catalase and peroxidase activities when treated with *Streptomyces* sp. IC10 and Y28, or with Y28 alone, respectively, outlining a strain-specific ISR in tomato against Fusarium wilt mediated by FOL (Abbasi et al., 2019). *Streptomyces bikiniensis* increased the activities of peroxidase, phenylalanine ammonia-lyase and β-1,3-glucanase in cucumber leaves (Zhao et al., 2012). Non-pathogenic *F. oxysporum* Fo47 can triger ISR to FOL and protect tomato from Fusarium wilt (Fuchs et al., 1999). *Trichoderma gamsii* IMO5 increased transcript levels of ISR-marker genes *ZmLOX10*, *ZmAOS* and *ZmHPL* in maize leaves, thereby protecting the plant from the pink ear rot pathogen *F. verticillioides* (Galletti et al., 2020).

An important determinant of biocontrol efficacy is the population density of ISR-triggering microorganisms. For example, ~10^5 CFU of *Pseudomonas defensor* (ex *fluorescens*) WCS374 per g of root are required for significant suppression of Fusarium wilt of radish (Raaijmakers et al., 1995). Another important feature of ISR in plants is that its effects are not only expressed at the site of induction but also in plant parts that are distant from the site of induction (Pieterse et al., 2014). For example, root-colonizing *Pseudomonas simiae* (ex *fluorescens*) WCS417r induced resistance in carnation, with phytoalexin accumulation in stems, and protected shoots from *F. oxysporum* (Van Peer et al., 1991). Priming of barley heads with *P. fluorescens* MKB158 led to changes in the levels of 1203 transcripts (including some involved in host defense responses), upon inoculation with pathogenic *F. culmorum* (Petti et al., 2010).

Competition for space and nutrients

In the case of competition, biocontrol of pathogens occurs when another microorganism is able to colonize the environment faster and use nutrient sources more efficiently than the pathogen itself, especially under limited conditions (Maheshwari, 2013; Legrand et al., 2017). Bacteria and fungi have the ability to compete with *Fusarium*, but the underlying mechanism of competition is sometimes unclear. For example, competition between non-pathogenic *F. oxysporum* strains and pathogenic *F. oxysporum* has been described, reducing disease incidence (Eparvier and Alabouvette 1994; Fuchs et al., 1999). Similarly, a non-aflatoxigenic *Aspergillus flavus* strain was found to outcompete a mycotoxin-producing *F. verticillioides* during colonization of maize (Reis et al., 2020). Competition may involve bacteria such as *Pseudomonas capeferrum* (ex *putida*) strain WCS358, which suppresses Fusarium wilt of radish (Lemanceau et al., 1993).

In some cases, traits involved in competition have been identified. In *P. putida* (Trevisan) Migula isolate Corvallis, competition for root colonization entails plant's production of agglutinin, and *P. putida* mutants lacking the ability to agglutinate with this plant glycoprotein showed

reduced levels of rhizosphere colonization and suppression of Fusarium wilt of cucumber (Tari and Anderson, 1988). *P. capeferrum* WCS358 suppresses Fusarium wilt of radish by competing for iron through the production of its pseudobactin siderophore (Lemanceau et al., 1993). In addition to bacteria, the fungus *Trichoderma asperellum* strain T34 can control the disease caused by FOL on tomato plants by competing for iron (Segarra et al., 2010).

Antagonism

Another important microbial mechanism to suppress plant pathogens is the secretion by beneficial microorganisms of various antifungal metabolites. They include antifungal secondary metabolites, sometimes termed antibiotics (e.g., fengycin, iturin, surfactin (Chen et al., 2018), fusaricidin and polymyxin (Zalila-Kolsi et al., 2016)), as well as VOCs (Volatile Organic Compounds; Zaim et al., 2016; Legrand et al., 2017) (Table 2). Extracellular lytic enzymes such as cellulase, chitinase, pectinase, xylanase (Khan et al., 2018), protease and glucanase (Saravanakumat et al., 2017), can also interfere with *Fusarium* growth or activity.

Table 2. Biocontrol agents, *Fusarium* pathogens, and biocontrol metabolites and enzymes.

Biocontrol agent	Pathogen	Biocontrol metabolites and enzymes	Reference
Bacillus subtilis	*F. oxysporum* *F. graminearum*	Cellulase, chitinase, pectinase, xylanase, protease, fengycins and surfactins	Zhao et al., 2014; Zalila-Kolsi et al., 2016; Noor Khan et al., 2018
Bacillus velezensis	*F. graminearum* *F. culmorum*	Fengycin B, iturin A, surfactin A and siderophores	Chen et al., 2018; Adeniji et al., 2019
Bacillus pumilus	*F. oxysporum*	Chitinolytic enzymes and antibiotic surfactin	Agarwal et al., 2017
Bacillus amyloliquefaciens	*F. graminearum*	Iturin and surfactin	Zalila-Kolsi et al., 2016
Brevibacillus fortis	*F. oxysporum*	Edeine	Johnson et al., 2020
Brevibacillus reuszeri	*F. oxysporum*	Chitinolytic enzymes	Masri et al., 2021
Burkholderia sp.	*F. oxysporum*	Phenazine-1-carboxylic acid	Xu et al., 2020
Chryseobacterium sp.	*F. solani*	VOCs	Tyc et al., 2015
Gluconacetobacter diazotrophicus	*F. oxysporum*	Antibiotic (pyoluteorin) and VOCs	Logeshwarn et al., 2011
Kosakonia arachidis	*F. verticillioides* *F. oxysporum*	Chitinase, protease, cellulase and endoglucanase	Singh et al., 2021
Lysobacter antibioticus	*F. graminearum*	VOCs	Kim et al., 2019
Paenibacillus polymyxa	*F. graminearum* *F. oxysporum*	Fusaricidin, polymyxin and VOCs	Raza et al., 2015; Zalila-Kolsi et al., 2016
Pseudomonas sp.	*F. verticillioides* *F. graminearum*	Antifungal antibiotics and fluorescent pigments	Pal et al., 2001
Streptomyces spp.	*F. oxysporum*	Antibiotic compounds, lipopeptin A and B	Cuesta et al., 2012; Wang et al., 2023
Trichoderma sp.	*F. oxysporum* *F. caeruleum*	Pyrones, koningins and viridins	Reino et al., 2008

Bacillota representatives (formerly *Firmicutes*), i.e., *Bacillus* and *Brevibacillus* species are highlighted in several studies as candidates for *Fusarium* biocontrol through production of antifungal metabolites (Palazzini et al., 2007; Zhao et al., 2014; Chen et al., 2018; Johnson et al., 2020). *B. subtilis* SG6 has the ability to produce fengycins and surfactins acting against *F. graminearum* (Zhao et al., 2014), whereas *Bacillus velezensis* LM2303 exhibits strong antagonistic activity against *F. graminearum* and significantly reduces FHB severity under field conditions (Chen et al., 2018). Genome mining of *B. velezensis* LM2303 identified 13 biosynthetic gene clusters encoding secondary metabolites and chemical analysis confirmed their presence. These metabolites included three antifungal metabolites (fengycin B, iturin A, and surfactin A) and eight antibacterial metabolites (surfactin A, butirosin, plantazolicin and hydrolyzed plantazolicin, kijanimicin, bacilysin, difficidin, bacillaene A and bacillaene B, 7-o-malonyl macrolactin A and 7-o-succinyl macrolactin A) (Chen et al., 2018). *Brevibacillus fortis* NRS-1210 produces edeine, a compound with antimicrobial activity, which inhibits chlamydospore germination and conidia growth in *F. oxysporum* f. sp. *cepae* (Johnson et al., 2020). *Pseudomonadota* representatives (formerly *Proteobacteria*) are also known for disturbing *Fusarium* growth or activity. Thin layer chromatography analysis showed that *Gluconacetobacter diazotrophicus* produces pyoluteorin, which is involved in the suppression of *F. oxysporum* (Logeshwarn et al., 2011), while *Burkholderia* sp. HQB-1 produces phenazine-1-carboxylic acid, which is efficient at controlling Fusarium wilt of banana, caused by *F. oxysporum* f. sp. *cubense* (Xu et al., 2020). *Pseudomonas* sp. EM85 was successful in suppressing disease caused by *F. verticillioides* and *F. graminearum*, by producing antifungal antibiotics and fluorescent pigments (Pal et al., 2001). Besides bacteria, *Trichoderma* fungi synthesize a number of secondary metabolites such as pyrones (which completely inhibit spore germination of *F. oxysporum*), koningins (which affect the growth of *F. oxysporum*) and viridin (which prevents the germination of spores of *F. caeruleum*) (Reino et al., 2008).

VOCs have recently received more attention, as they can enable interactions between organisms in the soil ecosystem through both water and air phases (de Boer et al., 2019). *Paenibacillus polymyxa* WR-2 produced VOCs when cultivated in the presence of organic fertilizer and root exudates. Among them, benzothiazole, benzaldehyde, undecanal, dodecanal, hexadecanal, 2-tridecanone and phenol inhibited mycelial growth and spore germination of *F. oxysporum* f. sp. *niveum* (Raza et al., 2015). *Chryseobacterium* sp. AD48 inhibited growth of *F. solani* through the production of VOCs (Tyc et al., 2015). VOCs produced by *Lysobacter antibioticus* HS124 enhanced mycelial development, but they also reduced sporulation and spore germination of *F. graminearum* at the same time (Kim et al., 2019). In addition, testing the antagonistic mechanisms of *Aspergillus pseudocaelatus* and *T. gamsii* revealed the presence of the VOCs 2,3,4-trimethoxyphenylethylamine, 3-methoxy-2-(1-methylethyl)-5-(2-methylpropyl) pyrazine, (Z)-9- octadecenamide, pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, thieno [2,3-c] pyridine-3-carboxamide,4,5,6,7-tetrahydro-2-amino-6-methyl- and hexadecanamide, which have an inhibitory activity against *F. solani* (Zohair et al., 2018).

Regarding extracellular lytic enzymes, *B. subtilis* 30VD-1 antagonized FOL by producing cellulase, chitinase, pectinase, xylanase and protease (Khan et al., 2018), while *Bacillus pumilus* synthesized a chitinolytic enzyme that reduced severity of disease caused by *F. oxysporum* on buckwheat under gnotobiotic conditions (Agarwal et al., 2017). *Brevibacillus reuszeri* inhibited the growth of *F. oxysporum* by producing chitinolytic enzymes (Masri et al., 2021). *Kosakonia arachidis* EF1 produced different cell-wall degrading enzymes, such as chitinases, proteases, cellulases and endoglucanases, which inhibited growth of *F. verticillioides* and *F. oxysporum* f. sp. *cubense*. Scanning electron microscopy revealed broken fungal mycelia surface and hyphae

fragmentation when these two pathogens were grown in the presence of *K. arachidis* EF1 (Singh et al., 2021).

Parasitism

Mycoparasitism is an ancient lifestyle, during which one fungus parasitizes another fungus (Kubicek et al., 2011). It involves direct physical contact with the host mycelium (Pal and McSpadden Gardener, 2006), secretion of cell wall-degrading enzymes and subsequent hyphal penetration (Viterbo et al., 2002). Mycoparasitic relationships can be biotrophic, where the host remains alive and the mycoparasitic fungus obtains nutrients from the mycelium of its partner, or necrotrophic, where the parasite contacts and penetrates the host, resulting in the death of the host and allowing the mycoparasite to use the remains of the host as a nutrient source (Jeffries, 1995). Several species of fungi are mycoparasitic, of which *Trichoderma* is the best described. Contact between the mycoparasitic fungi *Gliocladium roseum*, *Penicillium frequentans*, *T. atroviride*, *T. longibrachiatum* or *T. harzianum* and their phytopathogenic targets *F. culmorum*, *F. graminearum* and *F. nivale* triggers the formation of various mycoparasitic structures, such as hooks and pincers, which lead to cell disruption in the phytopathogens (Pisi et al., 2001). When *T. asperellum* and *T. harzianum* were grown in the presence of *F. solani* cell wall, they secreted several cell wall-degrading enzymes, such as β-1,3-glucanase, *N*-acetylglucosaminidases, chitinase, acid phosphatase, acid proteases and alginate lyase (Qualhato et al., 2013), and similarly, *Clonostachys rosea* produced chitinase and β-1,3-glucanase in the presence of *F. oxysporum* cell wall (Chatterton and Punja, 2009). *Sphaerodes mycoparasitica* is a biotrophic fungus that parasitizes *F. avenaceum*, *F. oxysporum* and *F. graminearum* hyphae and forms hooks as parasitic structures (Vujanović and Goh, 2009). However, the direct contribution of mycoparasitism to biological control is difficult to quantify as mycoparasitic fungi typically exhibit a number of different biocontrol mechanisms (Pal and McSpadden Gardener, 2006).

Inhibition and detoxification of mycotoxins

Biocontrol research often focuses on pathogen inhibition, and effects on mycotoxin synthesis or detoxification are often neglected (Pellan et al., 2020). It can be expected that *Fusarium* inhibition will diminish mycotoxin synthesis, but one comprehensive study found that *B. amyloliquefaciens* FZB42 inhibited *F. graminearum* but at the same time stimulated biosynthesis of DON toxin (Gu et al., 2017). Conversely, DON production of *F. graminearum* (on wheat kernels) was reduced by more than 80% with *B. amyloliquefaciens* WPS4-1 and WPP9 (Shi et al., 2014), and *Paenibacillus polymyxa* W1-14-3 and C1-8-b (He et al., 2009), whereas *Pseudomonas* strains MKB158 and MKB249 significantly reduced DON production in *F. culmorum*-infected wheat seeds (Khan and Doohan, 2009). *Pseudomonas* sp. MKB158 lowered expression of the gene coding for trichodiene synthase (an enzyme involved in the production of trichothecene mycotoxins in *Fusarium*) by 33%, in wheat treated with *F. culmorum* (Khan et al., 2006). DON production in both *F. graminearum* and *F. verticillioides* was also inhibited by the fungus *T. asperellum* TV1 and the oomycete *Pythium oligandrum* M1/ATCC (Pellan et al., 2020). Other mycotoxins may be targeted, as *T. harzianum* Q710613, *T. atroviride* Q710251 and *T. asperellum* Q710682 decreased ZEA production in a dual-culture assay with *F. graminearum* (Tian et al., 2018), and *Streptomyces* sp. XY006 lowered the synthesis of fusaric acid in *F. oxysporum* f. sp. *cubense* (Wang et al., 2023).

SOILS SUPPRESSIVE TO *FUSARIUM* DISEASES

Soils suppressive to *Fusarium* diseases and comparison with other suppressive soils

The phenomenon of disease suppressiveness has been described for many soil-borne fungal pathogens, including *Gaeumannomyces graminis* var. *tritici* (Shipton et al., 1973), *Thielaviopsis basicola* (Stutz et al., 1986) and *Rhizoctonia solani* (Mendes et al., 2011). It is also well established in the case of several *Fusarium* pathogenic species (Table 3), such as *F. culmorum* on wheat (in the Netherlands and Germany; Ossowicki et al., 2020) and barley (in Denmark; Rasmussen et al., 2002), *F. oxysporum* f. sp. *albedinis* on palm tree (in Marocco; Rouxel and Sedra, 1989), *F. oxysporum* f. sp. *batatas* on sweet potato (in California; Smith and Snyder, 1971), *F. oxysporum* f. sp. *cubense* on banana (in India, Indonesia, China, Gran Canaria island and several Central America states; Stotzky and Torrence Martin, 1963; Domínguez et al., 1996; Shen et al., 2015b; Nisrina et al., 2021; Yadav et al., 2021), *F. oxysporum* f. sp. *cucumerinum* on cucumber (in California; Sneh et al., 1984), *F. oxysporum* f. sp. *dianthi* on carnation (in Italy; Garibaldi et al., 1983), *F. oxysporum* f. sp. *fragariae* on strawberry (in Korea; Cha et al., 2016), *F. oxysporum* f. sp. *lini* on flax (in Italy, California; Kloepper et al., 1980; Tamietti and Pramotton, 1990), FOL on tomato (in France, Italy; Tamietti and Alabouvette, 1986; Tamietti et al., 1993) and wheat (in Italy; Tamietti and Matta, 1984), *F. oxysporum* f. sp. *melonis* on melon (in France; Louvet et al., 1976), *F. oxysporum* f. sp. *niveum* on watermelon (in Florida; Larkin et al., 1993), *F. oxysporum* f. sp. *radicis-cucumerinum* on cucumber (in Israel; Klein et al., 2013), *F. udum* on pigeon-pea (in India; Vasudeva and Roy, 1950), *F. graminearum* on wheat (in Serbia; Todorović et al., submitted; Chapter 2) and in a soil fungistasis context (in France; Legrand et al., 2019). Therefore, unlike with other pathogenic taxa, suppressiveness is documented across a wide range of *Fusarium* pathosystems. It also appears that suppressiveness to *Fusarium* diseases occurs in numerous parts of the world (Figure 3).

Table 3. List of locations with soils suppressive to *Fusarium* diseases known to date, with a pathosystem, disease and the underlying suppression mechanism.

Pathogen	Disease	Country	Suppression mechanism	References
F. culmorum	Seedling blight of barley	Denmark	Soil microbiota that has a more efficient cellulolytic activity	Rasmussen et al., 2002
F. culmorum	*F. culmorum* disease in wheat	Netherlands and Germany	No specific taxa, but a guild of bacteria working together	Ossowicki et al., 2020
F. graminearum	No disease supression tested, only fungistasis	Britanny, France	*Pseudomonas* and *Bacillus*	Legrand et al., 2019
F. graminearum	Wheat damping-off	Serbia	Under progress	Todorović et al., submitted
F. oxysporum f. sp. *albedinis*	Bayoud vascular wilt of palm tree	Marocco	Competition with soil microbiota	Rouxel and Sedra, 1989
F. oxysporum f. sp. *melonis*	Fusarium wilt of watermelon	Châteaurenard, France	Competition with soil microbiota including non-pathogenic *Fusarium*	Louvet et al., 1976; Alabouvette et al., 1985
F. oxysporum f. sp. *fragariae*	Fusarium wilt of strawberry	Korea	*Streptomyces*, wilt-suppressive soil that was developed through monoculture	Cha et al., 2016
F. oxysporum f. sp. *dianthi*	Vascular wilting disease of carnations	Albenga, Italy	Competition with other *Fusarium* strains	Garibaldi et al., 1983
F. oxysporum f. sp. *batatas*	Fusarium wilt on sweet potato	California, USA	No data	Smith and Snyder, 1971
F. oxysporum f. sp. *cubense*	Banana wilt disease	Ayodhya district, India	*Bacillus licheniformis* producing antifungal secondary metabolites	Yadav et al., 2021
F. oxysporum f. sp. *cubense*	Banana wilt disease	Gran Canaria, Spain	Sodium in soil	Domínguez et al., 1996
F. oxysporum f. sp. *cubense*	Banana wilt disease	Indonesia	*Pseudomonas* and *Burkholderia*	Nisrina et al., 2021
F. oxysporum f. sp. *cubense*	Banana wilt disease	Honduras, Costa Rica, Panama and Guatemala	Clay mineralogy, presence of montmorillonite-type clay in suppressive soil	Stotzky and Torrence Martin, 1963

F. oxysporum f. sp. *cubense*	Banana wilt disease	Hainan, China	*Pseudomonas* inducing jasmonate and salicylic acid pathways and shared core microbiome in suppressive soils	Shen et al., 2015b; Zhou et al., 2019; Shen et al., 2022; Wang et al., 2022; Lv et al., 2023
F. oxysporum f. sp. *cucumerinum*	Fusarium wilt of cucumber	California, USA	*Pseudomonas* siderophores and lytic bacteria	Sneh et al., 1984
F. oxysporum f. sp. *lini*	Fusarium wilt of flax	California, USA	*Pseudomonas* siderophores	Kloepper et al., 1980
F. oxysporum f. sp. *lini*	Fusarium wilt of flax	Carmagnola and Santena, Italy	Competition with other *Fusarium*	Tamietti and Pramotton, 1990
F. oxysporum f. sp. *lycopersici*	Fusarium wilt of tomato	Noirmoutier, France	Non-pathogenic *F. oxysporum*	Tamietti and Alabouvette, 1986
F. oxysporum f. sp. *lycopersici*	Fusarium wilt of wheat	Albenga, Italy	Non-pathogenic *F. oxysporum* inducing plant defense	Tamietti and Matta, 1984
F. oxysporum f. sp. *lycopersici*	Fusarium wilt of tomato	Albenga, Italy	Non-pathogenic *F. oxysporum* inducing plant defense	Tamietti et al., 1993
F. oxysporum f. sp. *niveum*	Fusarium wilt of watermelon	Florida, USA	Wilt-suppressive soil that was developed through monoculture	Larkin et al., 1993
F. oxysporum f. sp. *radicis-cucumerinum*	Cucumber crown and root rot	Israel	Suppresiveness induced by mixing sandy soil with wild rocket (*Diplotaxis tenuifolia*) debris under field conditions	Klein et al., 2013
F. udum Butl.	Wilt of pigeon-pea	Delhi, India	Soil microbiota	Vasudeva and Roy, 1950

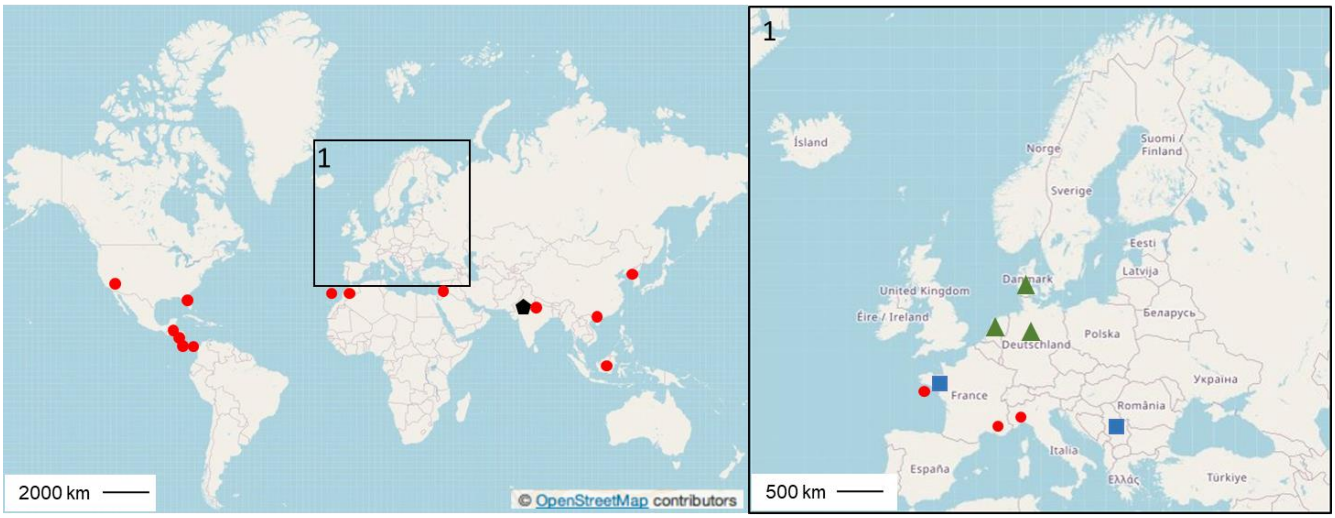

Figure 3. Geographic location of European field locations (map on the right) with soils suppressive to *Fusarium* diseases. In France (Noirmoutier island, Châteurenard in South-East France, and Brittany), Denmark, The Netherlands, Germany, Italy (Albenga, Carmagnola and Santena), Gran Canaria Island (Spain, located in the Atlantic ocean) and Serbia, with the corresponding pathogen i.e., *Fusarium oxysporum* (red dot), *Fusarium culmorum* (green triangle), or *Fusarium graminearum* (blue square). Soils suppressive to *Fusarium oxysporum* were also found outside Europe (map on the left), i.e., in North America: in California and Florida; Central America: in Honduras, Costa Rica, Panama and Guatemala; in Asia: in Korea, China, India, Israel and Indonesia; and in Africa: in Marocco. Soils suppressive to *Fusarium udum* were found in India (black pentagon).

In suppressiveness, the focus is put on the disease (whose extent is controlled by microbial populations, typically on roots), but pathogen suppressiveness i.e., the inability of the pathogen to survive and proliferate in soil can also take place. Fungistasis, which often entails competition with the rest of the soil microbiota, along with microbial release of inhibitors, is important for general suppression of many soil-borne fungal diseases (Garbeva et al., 2011; de Boer et al., 2019). Fungistasis can affect *Fusarium* pathogens (de Boer et al., 2019; Legrand et al., 2019), but its significance in relation to different *Fusarium* species or *formae speciales* needs clarification. Legrand et al. (2019) determined the soil fungistasis status of 31 wheat fields in the case of *F. graminearum*, highlighting higher bacterial diversity, a higher prevalence of *Pseudomonas* and *Bacillus* species and a denser network of co-occurring bacterial taxa in soils with fungistasis. It suggests the importance of cooperations within diversified bacterial communities to control *F. graminearum* in soil (Legrand et al., 2019). Accordingly, both bacterial and fungal communities differed between Fusarium wilt-diseased soils vs. healthy (presumably suppressive) soils taken from eight countries and grown with different crop plants (Yuan et al., 2020).

In addition to soil fungistasis, specific disease suppressiveness to *Fusarium* diseases may take place, thereby plants susceptible to *Fusarium* pathogen(s) show no or limited symptoms. Such suppressiveness is sometimes an intrinsic property of the soil and persists over years, despite changing ecological conditions related to crop rotation. This natural/long-term suppressiveness is well documented for several pathosystems, for instance in Swiss soils suppressive to tobacco black root rot (*T. basicola*) near Morens (Stutz, 1986). Suppressive and conducive soils may be located at small geographic distances in the landscape, and differences in

plant disease incidence between neighbouring fields that share similar climatic conditions and agronomic practices are attributed by the differences in the resident microbiota in these soils (Almario et al., 2014). Natural suppressiveness has also been extensively studied in the case of *Fusarium* diseases, in particular with the Fusarium wilt suppressive soils of Salinas Valley (California) or Châteaurenard (France). In these soils, Fusarium wilt disease remains minor despite the long history of cultivation of different crops, and the introduction of small amount of these soils to sterilized suppressive soil or conducive soil significantly decreased Fusarium wilt disease incidence (Scher and Baker, 1980; Alabouvette, 1986). In both locations, the small level of disease in plants cannot be attributed to the absence of *Fusarium* in the soil, but rather to plant protection by the soil microbiota (Sneh et al., 1984; Alabouvette et al., 1985).

Specific disease suppressiveness can also result from particular farming practices leading to the built-up of a plant-protecting microbiota. Often, this takes place following crop monoculture, typically after early disease outbreak, and is examplified by take-all decline of wheat (Weller et al., 2002; Sanguin et al., 2009) and barley (Schreiner et al., 2010), where suppressiveness is initiated and maintained by monoculture, in the presence of the pathogen *G. graminis* var. *tritici* (Weller et al., 2002). Soil suppressiveness to *Fusarium* diseases is usually natural, but cases of induced suppressiveness are also documented. Thus, soils found in Hainan island (China) that were grown for years with banana in confontration with pathogenic *F. oxysporum* displayed rhizosphere enrichment in microbial taxa conferring protection from banana wilt (Shen et al., 2022), watermelon monoculture in Florida induced suppressiveness to wilt caused by *F. oxysporum* f. sp. *niveum* (Larkin et al., 1993), and 15 years of strawberry monoculture in Korea triggered suppressiveness to wilt caused by *F. oxysporum* f. sp. *fragariae* (Cha et al., 2016). Soil addition of wild rocket residues resulted in suppressiveness to cucumber crown and root rot (*F. oxysporum* f. sp. *radicis-cucumerinum*) in Israel (Klein et al., 2013), whereas suppressiveness to Fusarium wilt can also be induced by microbial biofertilizer inoculants reshaping the soil microbiome (Xiong et al., 2017). Thus, organic fertilizer containing *B. amyloliquefaciens* W19 enhanced levels of indigenous *Pseudomonas* and provided suppression of Fusarium wilt of banana (Tao et al., 2020). The combined action of *B. amyloliquefaciens* W19 and *Pseudomonas* is thought to cause a decrease in *Fusarium* density in the root zone of banana. Organic fertilizers inoculated with *Erythrobacter* sp. YH-07 controlled Fusarium wilt in tomato, as a direct result of the bacteria and indirectly by altering the composition of the microbial community (Tang et al., 2023). Organic fertilizer amended with *Bacillus* and *Trichoderma* resulted in an increase in indigenous *Lysobacter*, thus indirectly inducing suppression of Fusarium wilt of vanilla (Xiong et al., 2017).

Many biocontrol strains originate from suppressive soils, and they were investigated as a mean to understand disease suppressiveness. In the case of *Fusarium* diseases, examples include *Pseudomonas* sp. Q2-87 (*P. corrugata* subgroup) (Weller et al., 2007), isolated from wheat in take-all decline soils but that protects tomato from *F. oxysporum* f. sp. *radicis-lycopersici*, *Pseudomonas* sp. C7 (*P. corrugata* subgroup) (Lemanceau and Alabouvette, 1991) and non-pathogenic *F. oxysporum* Fo47 (Fuchs et al., 1997; Duijff et al., 1998; Fuchs et al., 1999), isolated from soil suppressive to Fusarium wilt of tomato. Based on the biocontrol traits thus identified, the corresponding microbial functional groups have been characterized in suppressive vs. conducive soils, using isolate collections, molecular fingerprints or sequencing. Fluorescent *Pseudomonas* bacteria, especially those producing the antifungal metabolite 2,4-diacetylphloroglucinol, have been extensively targeted in take-all-decline soils (Cook and Rovira, 1976; Weller et al., 2002; Weller et al., 2007) and soils suppressive to black root rot (Stutz, 1986; Laville et al., 1992; Kyselková and Moënne-Loccoz, 2012), whereas studies on soils suppressive

to *R. solani* disease have focused on *Pseudomonas* producing antifungal lipopeptides (Mendes et al., 2011), and *Streptomyces* (Cordovez et al., 2015) and *Paraburkholderia graminis* (Carrión et al., 2018) producing volatile metabolites. In the case of soils suppressive to *Fusarium* diseases, competition with pathogenic *Fusarium* species is considered important, involving the entire soil microbiota or more specifically non-pathogenic *Fusarium* strains in Châteaurenard soils (Louvet et al., 1976; Alabouvette, 1986), or fluorescent *Pseudomonas* (iron competition; Scher and Baker, 1980; Sneh et al., 1984) in soils of Salinas Valley (California) or Châteaurenard (France). The role of extracellular lytic enzymes can be significant, as soil microbiota may protect barley from *F. culmorum* via a more efficient cellulolytic activity than the pathogen, which consequently is outcompeted for nutrients (Rasmussen et al., 2002). In banana, suppressiveness results in part from chitinolytic effects of the soil microbiota against the pathogen (Lv et al., 2023). Other modes of action include the production of antifungal secondary metabolites in wilt-suppressive soils, such as a new thiopeptide by *Streptomyces* (Cha et al., 2016) and phenazines by *Pseudomonas* (Mazurier et al., 2009), and immunity stimulation in banana (induction of the jasmonate and salicylic acid pathways) by fluorescent *Pseudomonas* (Lv et al., 2023).

Specific disease suppressiveness is attributed to the contribution of a few plant-benefical populations, but comparison of suppressive vs. conducive soils has evidenced differences in the occurrence or prevalence of multiple taxa, in the case of suppressiveness to take all (Sanguin et al., 2009; Schreiner et al., 2010; Chng et al., 2015), black root rot (Kyselková et al., 2009), *R. solani*-mediated damping-off (Mendes et al., 2011), or potato common scab (Rosenzweig et al., 2012). Similar findings were made with soils suppressive to *Fusarium* diseases. No single phylum was uniquely associated with *F. oxysporum* wilt suppressiveness in Korean soils, even though *Actinobacteria* was identified as the most prevalent bacterial taxa colonizing strawberry in suppressive soils (Cha et al., 2016). Likewise, the bacterial genera *Devosia*, *Flavobacterium* and *Pseudomonas* were more abundant (and the pathogen less abundant) in Chinese soils suppressive to banana wilt compared to conducive soils, and *Pseudomonas* inoculants isolated from suppressive soils could control the disease (Lv et al., 2023). Compared with conducive soil, Fusarium wilt suppressive soil from Châteaurenard displayed higher relative abundance of *Adhaeribacter*, *Arthrobacter, Amycolatopsis*, *Geobacter, Massilia*, *Microvirga*, *Paenibacillus*, *Rhizobium*, *Rhizobacter*, *Rubrobacter* and *Stenotrophomonas* (but not *Pseudomonas*) (Siegel-Hertz et al., 2018). However, differences were also found in the fungal community, with several fungal genera (*Acremonium*, *Ceratobasidium*, *Chaetomium*, *Cladosporium*, *Clonostachys*, *Mortierella*, *Penicillium*, *Scytalidium*, *Verticillium*, but also *Fusarium*) detected exclusively in the wilt suppressive soil (Siegel-Hertz et al., 2018). Data also pointed to a greater degree of microbial complexity in suppressive soils, with particular co-occurrence networks of taxa (Bakker et al., 2014; Lv et al., 2023). In German and Dutch soils, co-occurrence networks showed that the suppressive soil microbiota involves a guild of bacteria that probably function together, and in two of the suppressive soils this guild is dominated by *Acidobacteria* (Ossowicki et al., 2020).

Many studies focused on a few, geographically-close soils, which does not provide a global view on the importance of microbial diversity. However, two studies have considered geographically diverse agricultural soils suppressive to Fusarium wilt. Various Chinese soils suppressive to banana wilt mediated by *F. oxysporum* were shown to share a common core microbiota, specific to suppressive soils, which included the genus *Pseudomonas* (Shen et al., 2022). In a wider range of soils from the Netherlands and Germany, soils suppressive to *F. culmorum*-mediated wilt of wheat did not display a specific bacterial species that correlated with suppressiveness (Ossowicki et al., 2020). There was no relation either with soil physicochemical composition (i.e., soil type, pH, contents in C, N, or bioavailable Fe, K, Mg, P) or field history, yet

suppressiveness was microbial in nature, as sterilizing suppressive soils made them become conducive. This suggests that each suppressive soil may harbor its own set of phytobeneficial bacteria, supporting the notion of functional redundancy between microbiomes, meaning that different microbiomes may share common functionalities despite taxonomic differences in the microbial actors involved (Lemanceau et al., 2017). Taken together, this might be explained by the fact that protection of wheat from *F. culmorum*-mediated wilt corresponds to a case of natural suppressiveness, where biogeographic patterns are probably important, whereas banana wilt disease-suppressive soils are induced by monoculture, with convergent effects resulting from similar banana recruitment across different soil types.

Environmental factors potentially influencing soil suppressiveness to *Fusarium* diseases

Environmental conditions in soil may influence *Fusarium* autecology, the composition and activity of the soil microbial community, the tripartite interaction between this microbiota, *Fusarium* pathogens and the plant, and ultimately the level of disease suppressiveness (Marshall and Alexander, 1960; Amir and Alabouvette, 1993; Mazzola, 2002; Czembor et al., 2015). Key environmental factors in this regard include soil physicochemical properties and weather conditions (Weber and Kita, 2010).

Early work on the suppressiveness of soils to vascular Fusarium diseases drew attention to the positive role of certain abiotic factors and, in particular, montmorillonite-type clays (Stover, 1956; Stotzky and Torrence Martin, 1963). In addition, higher clay contents may contribute to reduced infestation by *Fusarium* (Kurek and Jaroszuk-Ściseł, 2003; Deltour et al., 2017), by altering oxygen diffusion, pH buffering and nutrient availability (Orr and Nelson, 2018). Höper et al. (1995) showed that the level of suppressiveness to Fusarium wilt of flax increased in soils amended with montmorillonite, kaolinite or illite at pH 7. A negative correlation between soil pH and *Fusarium* disease severity was reported in experiments with flax (Senechkin et al., 2014), strawberry (Fang et al., 2012) and banana (Shen et al., 2015a). Contrarily, the correlation between pH and Fusarium wilt incidence was positive in studies on banana (Peng et al., 1999) and watermelon (Cao et al., 2016). These inconsistencies may relate to the complexity of pH effects on *Fusarium* pathogens and diseases, and possible interactions with soil properties, *Fusarium* and plant genotypes, or other experimental conditions. In addition, soil suppressiveness to Fusarium wilt necessitates sufficient levels of nitrogen, as disease incidence negatively correlates with the NH_4^+ and NO_3^- contents in the soil (Li et al., 2016; Meng et al., 2019). Moreover, the addition of calcium to the soils suppressed Fusarium wilt in several soil types × plant conditions (Spiegel et al., 1987; Peng et al., 1999; Gatch and du Toit, 2017). In Brittany, *F. graminearum* growth positively correlated with manganese and iron contents in the soil (Legrand et al., 2019), while a positive correlation was also found between hemicellulose concentration and suppression of Fusarium wilt in tomato and carnation (Castaño et al., 2011), as well as cellulose concentration and suppression of Fusarium seedling blight of barley (Rasmussen et al., 2002). The two latter are attributed to the activity of cellulolytic microorganisms that limit *Fusarium* growth, as lower organic matter content (following decomposition) would reduce resources supporting this microbiota and disease suppression (Orr and Nelson, 2018).

Climatic conditions, notably temperature and precipitation may strongly affect the incidence of *Fusarium* diseases (Orr and Nelson, 2018). Phytopathogenic species *F. oxysporum*, *F. solani*, *F. verticillioides*, *F. graminearum* and *F. culmorum* develop best under humid conditions, at water activity above 0.86 (Table S1) (Thrane, 2014). Severity of Fusarium wilt in lettuce (Scott et

al., 2009; Ferrocino et al., 2013) and FHB in wheat is also positively correlated with soil temperature (Xu et al., 2007; Nazari et al., 2018). For example, Fusarium wilt incidence significantly increased when lettuce was grown at 22-26°C instead of 18-22°C (Ferrocino et al., 2013). Similarly, severity of Fusarium wilt of banana was significantly increased when temperature was raised from 24°C to 34°C (Peng et al., 1999).

Farming practices and their effects on soils suppressive to *Fusarium* diseases

As many other soil-inhabiting pathogenic fungi, *Fusarium* can overwinter as mycelium in plant debris or dormant structures in the soil, causing the initial infection of plants in the following season (Nelson et al., 1994; Janvier et al., 2007; Leplat et al., 2013; Xu et al., 2021). Therefore, cultural practices removing the primary inoculum of the pathogen from overwintering soils are useful to prevent future infection (Voigt, 2002). However, farming practices also influence soil suppressiveness by shaping the rhizosphere microbial community (Campos et al., 2016) and stimulating the activity of beneficial rhizosphere microorganisms (Janvier et al., 2007). In this context, various agricultural practices, such as crop rotation/monocropping, organic amendments, tillage, and fertilizers, are important to consider to develop suppressiveness-based control methods in farm fields (Janvier et al., 2007).

Except in the few cases when monoculture induces suppressiveness to *Fusarium* diseases (Larkin et al., 1993; Shen et al., 2022), cropping systems based on rotation of different plant species result in reduced survival of soil-borne pathogen propagules over the short term (Winter et al., 2014). Crop rotation may reduce severity and incidence of diseases caused by *Fusarium* (Wang et al., 2015; Khemir et al., 2020). For example, compared with the tomato monoculture, soil management under wheat-tomato rotation changes soil microbial composition by increasing the abundance of microbial taxa such as *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*, *Aspergillus*, *Penicillium* and *Mortierella*, which may control Fusarium wilt of tomato (De Corato et al., 2020). Reduced incidence of *F. pseudograminearum* and *F. culmorum* in the soils under cereal-legumes rotation management may be due to the non-host character of the legumes (Evans et al., 2010). However, not all crop rotations lead to reduced disease pressure (Ranzi et al., 2017). In the case of the FHB, it was advocated to rotate wheat and corn with crops like soybean, until it was shown that *F. graminearum* can also cause disease in soybean, as it has a wide range of hosts (Marburger et al., 2015). This suggests that there is no common rule regarding the relationship between crop rotation and *Fusarium* disease incidence.

Crop residues of high cellulose content promoted the activity of beneficial cellulolytic microorganisms and limited the development of *F. culmorum* (Rasmussen et al., 2002), as organic amendments represent a favorable environment for beneficial microorganisms that are able to combat phytopathogenic *Fusarium* species (Maher et al., 2008; Cuesta et al., 2012). Accordingly, organic amendments like animal manure, solid wastes and different composts are often used to improve soil health by delivering nutrients to the soil and also by stimulating beneficial microbiota (Mousa and Raizada, 2016). Thus, soils with added organic amendments exhibited inhibitory effects against *F. verticillioides* by reducing the production of fungal pigment and sporulation, consequently disabling fungal spread (Nguyen et al., 2018). Addition of vermicompost reduced tomato infection by FOL (Szczech, 1999) and mulched straw contributed to the suppression of seedling blight caused by *F. culmorum* (Knudsen et al., 1999). Soils supplemented with coffee residue compost or rapeseed meal exhibited suppressiveness to *F. oxysporum*-mediated wilt, and microorganisms isolated from supplemented soils inhibited *F. oxysporum* growth on agar plates (Mitsuboshi et al., 2018). Carbon addition to soil influenced the

soil microbiome, by enhancing the *Fusarium*-inhibitory populations from the *Streptomyces* genus (Dundore-Arias et al., 2020). However, increasing organic matter content may promote *Fusarium* survival in certain (rare) cases. One study tested the effects of 18 composts (made from different mixtures of manure, domestic biowaste and green waste) on Fusarium wilt disease suppression, caused by *F. oxysporum* f. sp. *lini*, and it was shown that only one compost did not positively affect the disease suppression (Termorshuizen et al., 2006). Overall, the efficiency of organic amendments in controlling plant diseases is determined by the pathosystem, the application rate, the kind of amendment and the level of maturity of composts or disintegration phase of crop residues (Janvier et al., 2007).

Tillage, which is one factor influencing crop residues decomposition, appears to have contrasting effects on soil suppressiveness. Under conventional tillage, tillage depth appears to play a crucial role in soil survival of *Fusarium*, such that the deeper the tillage, the lower the abundance of *Fusarium* species (Steinkellner and Langer, 2004). This can be partly explained by the fact that the pathogen is displaced from its niche, reducing its ability to survive (Bailey and Lazarovits, 2003), and the rate of decomposition of buried residues is faster than at the soil surface (Leplat et al., 2013). The carbon released during these decomposition processes increases the activity of the soil microbiota, thereby improving the overall functioning of the soil (Bailey and Lazarovits, 2003). Under conservation tillage, surface residues persist and can act as a long-term source of inoculum for plant infection by *F. verticillioides*, *F. proliferatum* and *F. subglutinans*, as they can colonise crop residues and produce overwintering spores that often survive the period when plants are absent from the agrosystem (Cotten and Munkvold, 1998; Bockus and Shroyer, 1998; Pereyra et al., 2004). This is consistent with results suggesting that conservation tillage and leaving crop residues *in situ* increase *Fusarium* abundance (Govaerts et al., 2008; Wang et al., 2020). For example, spores of *Fusarium* species could be recovered from plant residues more than two years after harvest (Pereyra et al., 2004). In certain cases, lower occurrence of plant infection by *F. culmorum*, *F. equiseti* (Weber et al., 2001) and *F. pseudograminearum* (Theron et al., 2023) was found under conservation tillage compared with conventional tillage. These contrasting results might be due to differences in *Fusarium* species, environmental factors and soil types. The use of simplified tillage practice was proposed to reduce *F. culmorum* abundance, by mixing crop residues with the topsoil layer to promote the growth of beneficial straw-decomposing microorganisms (Weber and Kita, 2010).

Different fertilizers have different effects on phytopathogenic *Fusarium*. On one hand, the development of FHB caused by *F. culmorum* and *F. graminearum* increased with inorganic nitrogen fertilization (Lemmens et al., 2004), and on the other hand, nitrite could reduce the population of *F. oxysporum* (Löffler et al., 1986). Besides, higher doses of nitrogen may contribute to higher accumulation of *Fusarium* mycotoxins (Podolska et al., 2017). The addition of phosphorus fertilizer, in the form of P_2O_5, significantly reduced *Fusarium*-caused wilting in chickpea, lentil and lupine, in both greenhouse and field conditions (Elhassan et al., 2010). Organic fertilizers can lead to an increase in indigenous microbial populations, thus contributing to suppression of Fusarium wilt disease (Montalba et al., 2010; Raza et al., 2015). When grown with the addition of organic N fertilizer, highbush blueberry exhibited increased tolerance to *F. solani*, in parallel to increased soil microbial activity and mycorrhizal colonization (Montalba et al., 2010).

CONCLUSION AND OUTLOOK

Disease-suppressiveness of soils is a useful model to understand microbial phytoprotection and develop sustainable plant protection strategies for soils devoid of this property. In this review, we summarized the current knowledge on *Fusarium* phytopathogens, the available control methods and soils suppressive to *Fusarium* diseases, with the underlying mechanisms involved in the suppression. On one hand, extensive information is available on environmental and microbial properties responsible for suppressiveness to *Fusarium* diseases. One prominent feature is the diversity of *Fusarium*-based pathosystems for which suppressive soils are documented, in terms of *Fusarium* species (often *F. oxysporum*, but not only), host plants (both monocots and dicots), types of disease (often wilt, but not only), geographic locations of soil and farming conditions, and types of suppressiveness (i.e., natural suppressiveness, but also monoculture-induced suppressiveness to *Fusarium* diseases, as well as fungistasis towards *Fusarium* pathogens). This diversity is paralleled by differences in microbiota composition and diversity associated with disease control in different cases of suppressiveness. On the other hand, despite the fact that soils suppressive to *Fusarium* diseases have been studied for decades, they are still poorly understood in terms of microbiota functioning, and knowledge remains fragmented.

On this basis, additional research is needed to integrate the scientific approaches used to decipher suppressiveness to *Fusarium* diseases. First, by combining complementary assessment methodology with current next-generation sequencing and ecological networks research, and incorporating experimental strategies to manipulate and transplant rhizosphere microbiome (or single microorganisms) of plants grown in suppressive soils to those in conducive soils to go beyond correlative work, as started recently (Ye et al., 2020; Jiang et al., 2022). Second, by extending the range of soil conditions investigated, and develop meta-analyses to estimate key microbiota differences between suppressive and conducive soils, as pioneered by Yuan et al. (2020). Third, by considering a wider range of biological actors, including beneficial fungi (often neglected) and soil fauna (likely to influence microbial communities, *Fusarium* vectorisation, and plant health; e.g., Dita et al., 2018; Wagner et al., 2022). Fourth, by taking into account plant genetics, behavior and physiological responses to *Fusarium* pathogens (e.g., Liu et al., 2019). Therefore, there is a need for a more multidisciplinary approach to understand microbiota functioning in soils suppressive to *Fusarium* diseases.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of this review article and approved the submitted version.

SUPPLEMENTARY DATA

Table S1. *Fusarium* species and optimum temperature, pH and water activity for growth.

Species	Optimum temperature	Optimum pH	Optimum a_w	Reference
F. oxysporum	25 to 27.5 ºC	5.1 to 5.9	> 0.89	Thrane, 2014; Jiménez-Díaz et al., 2015
F. solani	~ 29 ºC	~ 7.5	> 0.90	Thrane, 2014; Mohsen et al., 2015; Yan and Nelson, 2020
F. verticillioides	~ 27 ºC	6 to 7	> 0.87	Thrane, 2014; Kumar et al., 2019
F. graminearum	25 to 30 ºC	~ 3.5	> 0.90	Thrane, 2014; Panwar et al., 2017
F. culmorum	~ 25 ºC	~ 5	> 0.87	Aleandri et al., 2007; Scherm et al., 2013; Thrane, 2014

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CHAPTER 2

Manure amendments and fungistasis, and relation with protection of wheat from *Fusarium graminearum*

AVANT-PROPOS

Much knowledge is now available on different microbial populations that may be involved in phytopathogen suppression, as well as on their potential modes of action (Kyselková & Moënne-Loccoz, 2012; Todorović et al., submitted; Chapter 1). However, there is still a lack of knowledge on the rhizosphere ecology of these populations and on their phytoprotective activities in the ecological conditions that exist in the rhizosphere. Disease-suppressive and fungistatic soils represent model for studying phytoprotective microbial activities in the rhizosphere *in situ*, and for understanding biotic and abiotic factors favoring them. Suppressive soils gather an abundance of beneficial microorganisms, potentially acting against various soil-borne pathogens (Morimura et al., 2020), whereas the term general disease suppression refers to the activity of the entire soil microbiota that is limiting development and/or survival of several pathogens (Termorshuizen & Jeger, 2008). When propagules of fungal pathogens are efficiently affected by the activity of the entire soil microbiota, the term fungistasis is used (Garbeva et al., 2011). As a complement to general disease suppression, there can be also specific disease suppression, which entails suppression of disease caused by specific pathogen(s), and is mediated by the activity of one or a few microbial populations (Termorshuizen & Jeger, 2008). So far, various attempts have been made aiming to improve crop yield, soil health and crop protection from phytopathogens, through microbiome manipulation (De Corato, 2020). This manipulation usually relies on agricultural practices, such as manure or compost amendments (Bender et al., 2016; De Corato, 2020), use of microbial inoculants or bio-organic fertilizers, i.e., fertilizers supplemented with one or more bacterial strains (El-Hassan & Gowen, 2006). Thus, the in depth understanding of the microbiota functioning and their interaction with host plants in the natural ecosystems is needed in order to correctly and efficiently manipulate the microbiome for sustainable agriculture (Compant et al., 2010).

The importance of controlling different phytopathogenic *Fusarium* species in agricultural systems, as well as the potential of soils suppressive to *Fusarium* diseases were discussed in Chapter 1, and previously reported by many authors (Tamietti & Matta, 1984; Tamietti & Pramotton, 1990; Rasmussen et al., 2002; Ossowicki et al., 2020). In the present chapter, our objective was to test if: (i) fungistasis is influenced by manure amendments, (ii) the soil fungistasis towards *F. graminearum* is linked to the soil physicochemical properties, (iii) fungistatic soils may also be suppressive to Fusarium damping-off disease of wheat, and if (iv) there are particular microbial diversity patterns in the rhizosphere of plants grown in suppressive vs. non-suppressive soils. Highly virulent fungal strain *F. graminearum* MDC_Fg1 was provided by Thierry Langin (GDEC, INRAE, Clermont-Ferrand, France). To achieve our objectives, we sampled 26 fields from northern and western/central Serbia (with contrasting regions in terms of geography, soil type, farming management (Nejgebauer et al., 1971; Tanasijević et al., 1964), and with contrasting history of manuring), and we sterilized one part of each soil, subsequently inoculating both sterilized and non-sterilized soils with *F. graminearum* inoculum and incubating them for 15 days in controlled conditions. Control was represented by the non-sterilized, non-inoculated soils. In order to assess the fungistatic (fungus-inhibiting) potential of these soils, at day 15, all of the soils were sampled, total soil DNA was extracted and a qPCR approach was used to quantify the amount of *F. graminearum* DNA present in both sterilized and non-sterilized soils after an incubation period, using *F. graminearum* - specific primers, according to a protocol developed by Legrand et al. (2019). Physicochemical analysis of all 26 soils was performed at Fruit Research Institute in Čačak (Serbia). Then, aiming to check if fungistatic soils may also be suppressive to damping-off disease of wheat, we chose 4 soils where fungistatic

potential was related to the addition of manure amendments, we re-sampled them and performed a greenhouse suppressiveness assay. For each of the 4 soils, 20 pots were filled with 50:50 mixture of soil and sterile sand, each pot was filled with 5 seeds of winter wheat (*Triticum aestivum* L.), and then each seed was inoculated with *F. graminearum* spore suspension. At 14 days, we measured number of germinated seeds, and at 28 days, we measured number of plants alive, shoot length (cm), dry shoot biomass (mg), and dry shoot density. Finally, rhizosphere of wheat plants from the greenhouse experiment were used to perform 16S rRNA and ITS metabarcoding, and to compare soils based on prokaryotic and fungal taxonomic composition and diversity.

All of this work has led to the drafting of publication "Manure amendments and fungistasis, and relation with protection of wheat from *Fusarium graminearum*" (submitted to *Applied Soil Ecology* in September, 2023).

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MANURE AMENDMENTS AND FUNGISTASIS, AND RELATION WITH PROTECTION OF WHEAT FROM *FUSARIUM GRAMINEARUM*

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ABSTRACT

Certain soils promote crop health because they are pathogen-suppressive (i.e., fungistatic) or disease-suppressive, but the effect of soil management on these properties is not fully understood. Here, we tested the hypothesis that manure could favor fungistasis by screening 26 manured and non-manured wheat fields from Serbia for their ability to control survival of *Fusarium graminearum* Fg1. *F. graminearum* Fg1 grew in all 26 soils if autoclaved. In the absence of autoclaving, qPCR showed that the pathogen was stable or grew in 16 soils (37% manured), but declined in the 10 others (70% manured). There was no global relation between soil chemistry and fungistasis, except in Mionica where fungistatic (and manured) soils MI2 and MI3 displayed higher organic matter and potassium contents than non-fungistatic (and non-manured) soils MI4 and MI5. Using soils from Mionica, we then tested the hypothesis that fungistatic (manured) soils rather than non-fungistatic (non-manured) soils would protect wheat from *F. graminearum* disease. Indeed, non-fungistatic soil MI4 was conducive to wheat damping-off but non-fungistatic soil MI5 was suppressive, whereas both fungistatic soils MI2 and MI3 were suppressive. Metabarcoding showed that the structure of prokaryotic and fungal rhizosphere communities depended mostly on the field of origin, with a significant effect of *F. graminearum* inoculation. Several prokaryotic and fungal taxa were soil specific, and pathogen inoculation changed community composition. In conclusion, our findings show that certain farming practices (here, manure amendments) may promote soil fungistasis towards *F. graminearum*. However, both fungistatic and non-fungistatic soils can be suppressive to *F. graminearum* disease in wheat, and their differences in rhizosphere microbiota suggest different phytoprotection mechanisms.

Keywords: pathogen suppression, disease suppression, fungistasis, rhizosphere, microbiome, metabarcoding

INTRODUCTION

Soil hosts a diversified community of microorganisms, which present beneficial, detrimental, or neutral effects on plants (Berendsen et al., 2012; Vacheron et al., 2013). The resulting impact on plant health and performance depends on multiple microbe-microbe and plant-microbe interactions. Within the complex rhizosphere ecosystem, these multiple interactions may lead to effective plant protection, despite the presence of virulent pathogen(s) and environmental conditions favorable for disease development. In soils where this emerging property takes place, plants exhibit limited or no disease symptoms and such soils are termed disease-suppressive soils (Hornby, 1983; Raaijmakers et al., 2009; Schlatter et al., 2017; Mitsuboshi et al., 2018).

Practically speaking, disease suppressiveness refers to the inherent ability of certain soils to actively restrict the population size, physiological activity, or negative effects of phytopathogens. While several studies attributed suppressiveness to particular soil physicochemical properties (Stotzky and Torrence Martin, 1963; Almario et al., 2014), the soil microbiome plays a prominent role (Mazurier et al., 2009; Almario et al., 2014; Ossowicki et al., 2020). Certain disease-suppressive soils differ from non-suppressive counterparts in terms of soil microbiota diversity (Kyselková et al., 2009; Legrand et al., 2019; Ossowicki et al., 2020).

Plant-protecting soil microorganisms may inhibit pathogens directly, through competition or antagonism, or indirectly by stimulating other plant-associated microorganisms or inducing plant immune responses (Mazzola, 2002; Raaijmakers et al., 2009). Often, these interactions take place in the rhizosphere, and thus the analysis of disease-suppressiveness has focused on rhizosphere interactions (Almario et al., 2014; Ossowicki et al., 2020). However, specific plant-beneficial interactions can be implemented in the soil itself, where they may lead to reduced saprophytic survival of the pathogen (Leplat et al., 2013; de Boer et al., 2019; Legrand et al., 2019), a property often referred to as fungistasis in the case of fungal pathogens (Garbeva et al., 2011; Sipilä et al., 2012; Legrand et al., 2019). Arguably, the effects of both fungistasis and rhizosphere-based disease-suppressiveness can be expected to add up in terms of phytoprotection efficacy, but these two aspects have rarely been considered together. Probably, soils with fungistasis would lead to particular microbial diversity patterns in the rhizosphere, with the potential to influence disease-suppression potential.

In this work, we tested the hypotheses that soil fungistasis is associated with specific soil physicochemistry, can be influenced by manure, leads to a particular microbial diversity pattern in the rhizosphere, and may promote rhizosphere-based disease-suppressiveness. To this end, we focused on *Fusarium graminearum* as a pathogen, because soils suppressive to *Fusarium* diseases have been evidenced in different geographic regions and *Fusarium* pathosystems (for review see Chapter 1), and this pathogen can be strongly affected by fungistasis (Legrand et al., 2019). We screened 26 agricultural soils from five locations in Serbia with contrasting soil properties (chernozems, pseudogleys, eutric cambisols, and vertisols) for their ability to inhibit the development of *F. graminearum*. Since agricultural practices such as organic amendments, fertilization, and crop rotation can impact the soil microbiota, with the potential to influence both soil fungistasis and disease-suppressiveness properties (Cuesta et al., 2012; Mousa and Raizada, 2016; Legrand et al., 2019; De Corato, 2020), we chose soils amended with manure and soils that did not receive manure. A selection of fungistatic and non-fungistatic soils was then assessed for suppressiveness to *F. graminearum*-mediated damping-off of wheat, and they were compared based on fungal and bacterial microbiota diversity in the wheat rhizosphere.

MATERIAL AND METHODS

Soil sampling

Soil sampling was conducted in 26 agricultural fields from five locations in Serbia, i.e., Sombor (SO) and Novi Karlovci (NK) in northern Serbia, and Valjevo (VA), Mionica (MI), and Čačak (CA) in western/central Serbia (Table 1; Figure 1A). Some fields received manure amendments regularly, but others did not (Table S1). Wheat was the predominant rotation crop in all fields. In each field, 6 areas at intervals of 10 meters were sampled in October 2020. The top few centimeters of soil were carefully removed, and soil samples were collected at a 5-20 cm depth. These individual samples were then combined to get one composite sample per field. Soils were then sieved (0.5 cm), and stones, roots and other organic material were removed.

Table 1. Locations, sample ID, manure amendments, soil types and GPS coordinates of 26 Serbian soils.

Location	Sample ID	Manure application	Soil type*	GPS coordinates	
Sombor	SO1	No	Chernozem	45.758696 N	19.1840320 E
	SO2	No	Chernozem	45.746168 N	19.159358 E
	SO3	Yes	Chernozem	45.750012 N	19.170019 E
	SO4	Yes	Chernozem	45.750839 N	19.172977 E
Novi Karlovci	NK1	Yes	Chernozem	45.060182 N	20.215013 E
	NK2	Yes	Chernozem	45.060066 N	20.215213 E
	NK3	No	Chernozem	45.088806 N	20.102067 E
	NK4	No	Chernozem	45.088011 N	20.099312 E
Valjevo	VA1	Yes	Eutric cambisol	44.33050 N	19.968102 E
	VA2	No	Eutric cambisol	44.330491 N	19.966663 E
	VA3	No	Eutric cambisol	44.330466 N	19.969106 E
	VA4	Yes	Eutric cambisol	44.330110 N	19.968102 E
	VA5	No	Pseudogley	44.351892 N	19.981415 E
	VA6	Yes	Pseudogley	44.351155 N	19.978144 E
	VA7	Yes	Pseudogley	44.355395 N	19.977465 E
	VA8	No	Pseudogley	44.355012 N	19.977650 E
Mionica	MI2	Yes	Vertisol	44.24611 N	20.10431 E
	MI3	Yes	Vertisol	44.24540 N	20.10350 E
	MI4	No	Vertisol	44.24745 N	20.10012 E
	MI5	No	Vertisol	44.24759 N	20.09931 E
Čačak	CA1	No	Vertisol	43.89897 N	20.54435 E
	CA2	Yes	Vertisol	43.89910 N	20.54450 E
	CA3	No	Vertisol	43.89905 N	20.54312 E
	CA4	Yes	Vertisol	43.89930 N	20.54315 E
	CA5	No	Vertisol	43.8867833 N	20.5462167 E
	CA6	Yes	Vertisol	43.8878667 N	20.5475167 E

* Based on Tanasijević et al. (1964) and Nejgebauer et al. (1971).

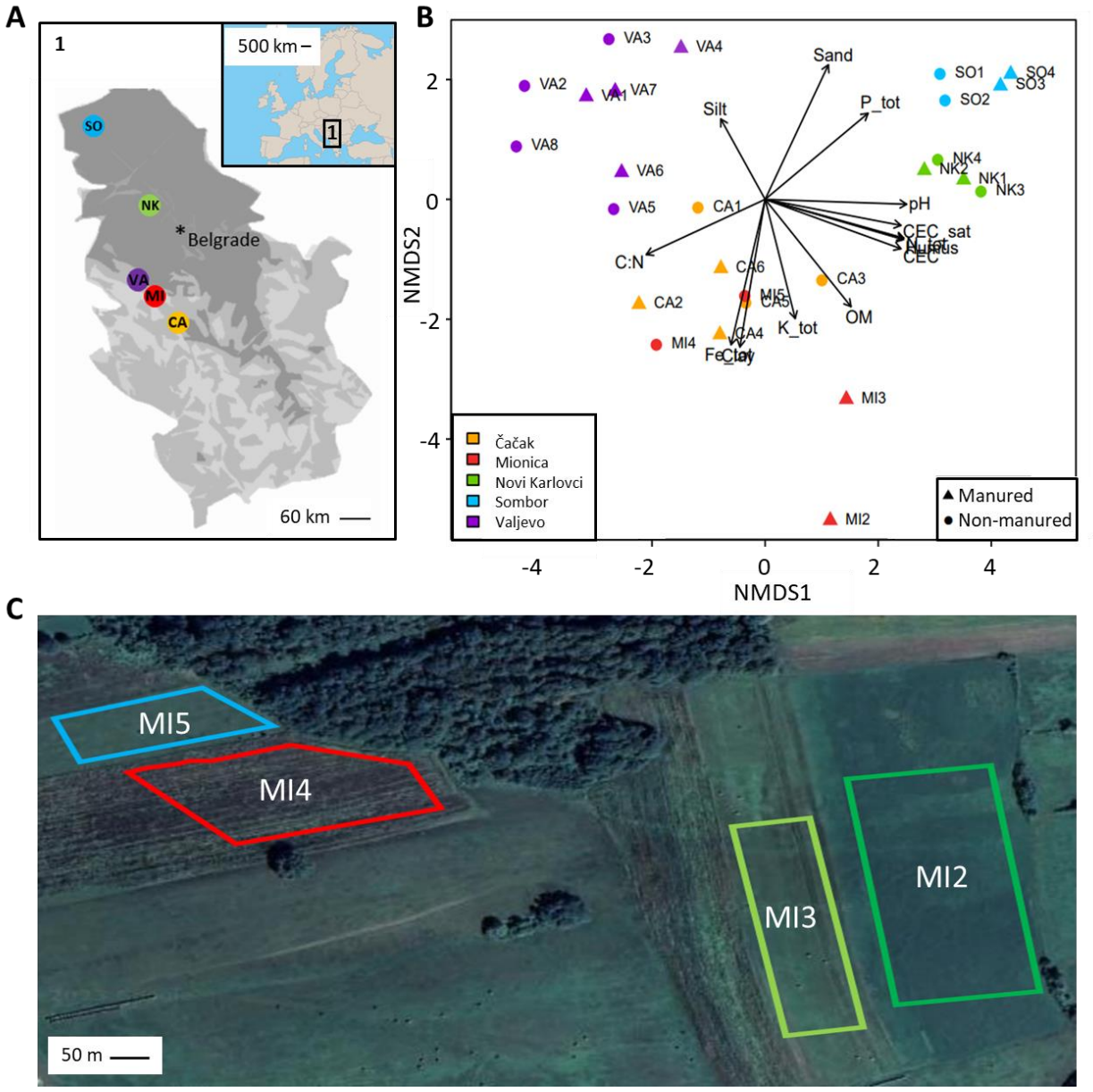

Figure 1. Soil sampling locations in Serbia. (A) Five locations in Serbia, i.e., Sombor (SO), Novi Karlovci (NK), Valjevo (VA), Mionica (MI) and Čačak (CA) where the 26 fields were sampled. (B) Non-Metric Multidimensional Scaling analysis of the physicochemical composition (see Table S2 for details) of the 26 Serbian fields. (C) Aerial picture of the four sampling fields MI2, MI3, MI4 and MI5 at Mionica, Serbia, visualized in Google Maps [Map data ©2023, Google].

Soil physicochemical analysis

Soil physicochemical analysis was carried out at the Fruit Research Institute in Čačak (Serbia). Mechanical properties of the soils were determined by dry sieving procedures, disaggregation with 4% $Na_4P_2O_7 \cdot 10H_2O$ and the pipetting method. Cation exchange capacity (CEC; cmol/kg) and CEC saturation (%) were determined using the Kappen method. Agrochemical soil determinations included pH in H_2O and contents in humus (Kotzmann method; %), organic

matter (combustion; %), total nitrogen (recalculated from humus content; %), readily available phosphorus (extraction with ammonium lactate; mg/kg), readily available potassium (extraction with ammonium lactate; mg/kg), and total Fe ($HCl:HNO_3$ 1:3; %).

Fusarium graminearum inoculum and spore suspension preparation

The virulent strain *Fusarium graminearum* MDC_Fg1 (hereafter Fg1), obtained from contaminated cereals in northern France (Alouane et al., 2018), was used in the experiments. To obtain mycelia used in the fungistasis experiment, the fungus was grown for eight days at 20-22°C on Potato Dextrose Agar (PDA; Conda Pronadisa, Madrid, Spain). To prepare the inoculum, a protocol adapted from Legrand et al. (2019) was followed. Maize grains were soaked in water 72 h at 22°C. They were then ground to Ø 1-2 mm and put into 2-l Erlenmeyer flasks, which were autoclaved two times for 20 min at 121°C with a 24-h interval. After autoclaving, inoculation was done with 7-mm-diameter plugs taken from the edge of 8-day-old PDA cultures of *F. graminearum* Fg1. The flasks were incubated 10 days at 22°C with vigorous shaking for 5 min once a day to promote kernel colonization, and colonized kernels were used as inoculum.

To obtain spore suspension used in the rhizosphere experiment, we used Mung Bean Broth (MBB) (Evans et al., 2000), which was prepared by adding 40 g of organic mung bean seeds in 1 l of boiling water and leaving to infuse and cool down for 10 min. After that, beans were discarded and 50 ml of the resulting medium was poured into 250-ml Erlenmeyer flasks and autoclaved for 20 min at 121°C. MBB (50 ml) was inoculated with ten 7-mm-diameter plugs taken from the edge of a 8-day-old *F. graminearum* Fg1 PDA plate and incubated for six days at 22°C and 180 rpm agitation (Incubator Shaker Series I26, New Brunswick Scientific, Edison, NJ, USA). At the sixth day, the preculture was diluted to one-tenth with fresh MBB and incubated under the same conditions for 10 days. The culture was filtered using sterile Miracloth to discard mycelium and centrifuged for 10 min at 4700*xg* (Avanti J-E Series, Beckman Coulter, Fullerton, CA, USA) at room temperature. The resulting pellet was washed twice with sterile water. Titration of spores in the suspension was performed using a Thoma counting chamber.

Evaluation of soil fungistasis to *Fusarium graminearum*

Prior to inoculation, four 1-g autoclaved samples and four 1-g non-autoclaved samples of each soil, as well as four 1-ml samples of *F. graminearum* Fg1 inoculum were collected and stored at -20°C before quantifying *F. graminearum* Fg1 DNA. The experiment was done in 20-ml vials containing 15 g soil, which was autoclaved (for 20 min at 121°C on two consecutive days) or not, and then inoculated (600 µl of mycelia inoculum) or not (600 µl of water), giving for each of the 26 soils (i) 4 inoculated, autoclaved vials, (ii) 4 inoculated, non-autoclaved vials, and (ii) 4 non-inoculated, non-autoclaved vials, i.e., 26 × (4 + 4 + 4) = 312 vials. The vials were arranged following a randomized block design and incubated in the dark at 60% air humidity and 20°C. Every three days, vials were weighted to estimate water loss, and the corresponding amount was added back. After 15 days, all soil samples were lyophilized (Lyophilizator, Alpha 1-4LSC, Christ, Germany) for 48 h, 1 g soil was sampled from each vial and stored at -20°C until DNA extraction.

Total DNA was extracted from 0.5 g soil for each of the 520 samples (208 samples before inoculation and 312 samples at 15 days) and from 1 ml of each Fg1 inoculum sample (4 samples), using FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch-Graffenstaden, France), according to manufacturer's instructions. *F. graminearum* Fg1 DNA was quantified by qPCR using a CFX-96TM Real-Time PCR System (Bio-Rad, Hercules, CA, USA). qPCR were performed in 20 µl containing 10

µl of SensiFAST SYBR No-ROX master mix (Bioline, Meridian Bioscience, Cincinnati, OH, USA), 1 µl of primers Fg16N-F (5'-ACAGATGACAAGATTCAGGCACA-3') and Fg16N-R (5'-TTCTTTGACATCTGTTCAACCCA-3') (Nicholson et al., 1998) at a final concentration of 20 µM, 6 µl of DNAse-free water and 2 µl of DNA sample. The qPCR cycle program consisted of 2 min denaturation at 98°C, and 40 cycles of denaturation at 98°C for 3 s and annealing/extension at 60°C for 3 s. All samples were run in triplicate, and negative controls were included to each run. Melting curve and Melting temperature (Tm) were determined using the Tm Calling Analysis module of LightCycler Software (v.1.5; Roche Applied Science, Meylan, France), and Cycle threshold (Ct) of each sample was determined with the second derivative maximum method in the LightCycler Software (v.1.5; Roche Applied Science). A melting curve was generated at the end of each qPCR run with a temperature gradient of 0.5 °C.s^{-1} from 60°C to 95°C (melting temperature of *F. graminearum* Fg1 amplicons was at 80°C). Only the amplicons with Tm ~ 80°C were considered as positive, while for all the amplicons with Tm different from 80°C, concentration of *F. graminearum* Fg1 in the sample was replaced by the quantification limit of 4.95×10^5 gene copies.g^{-1} dry soil.The standard curve was generated by plotting the mean Ct value of the three replicates (per DNA concentration) against DNA concentration. Amplification efficiency (E), calculated as $E = 10^{(1/slope)} - 1$, and the Mean Squared Error (MSE) of the standard curve were determined. Quantification of amplicons was achieved using a standard curve generated from serial dilutions (in three replicates) of previously extracted *F. graminearum* Fg1 genomic DNA ranging from 1.77×10^{-8} g.$µl^{-1}$ to 1.77×10^{-11} g.$µl^{-1}$. Results obtained in g.$µl^{-1}$ were transformed into numbers of copies.g^{-1} soil using the formula [DNA (g) × Avogadro's number (molecules.mol^{-1})] / [number of DNA matrix bp in amplified fragments × 660 (g.mol^{-1})], based on an average of 660 g.mol^{-1} per base pair. They were normalized to the total DNA quantity extracted from 0.5 g of soil and expressed into a number of copies.g^{-1} dry soil as previously done (Bouffaud et al., 2016). The amount in the Fg1 inoculum was calculated for 1 ml (same calculation as for 1 g of soil), extrapolated to the 600 µl used to inoculate 15 g of soil, and expressed per g of soil. This amount was subtracted from the DNA quantity found in each sample of 1 g of soil. All results were log_{10}-transformed for subsequent analysis. Mean values and standard deviation were calculated. The fungistasis level was computed according to a formula adapted from Legrand et al. (2019):

$$\Delta day15 = \log10(\text{FgDNA in soil at day 15 after inoculation}) - \log10(\text{FgDNA in the inoculum})$$

Wheat damping-off suppressiveness assay

The wheat damping-off suppressiveness assay with *F. graminearum* Fg1 was conducted with the four MI soils (Figure 1C), in a plant growth chamber (FitoClima, 10.000 EH, ARALAB, Rio de Mouro, Portugal) with 16 h day at 20°C, 8 h dark period at 18°C and relative humidity of 80%. Soils had been collected in June 2021. For each soil, 100 seeds of winter wheat (*Triticum aestivum* L.) variety Récital were distributed into 20 pots (height 12 × 10 × 10 cm; 5 seeds per pot) filled with 250 g of soil mixed with sterile siliceous sand (granulometry 0.6-1.6 mm, Gedimat, Dagneux, France; autoclaved twice, at 24 h interval) in a 50:50 ratio. In 10 pots, the seeds were inoculated with 100 µl of spore suspension (10^6 spores per seed), while seeds in the other 10 pots received 100 µl of water (control). The experiment followed a randomized block design with 10 blocks (n = 10). The plants were watered every 3 days by adding water under each pot, to maintain water content close to 21% w/w.

The number of germinated seeds was recorded at 14 days, and (i) the number of plants alive, (ii) shoot length (cm), (iii) dry shoot biomass (mg), and (iv) dry shoot density (i.e., shoot length divided by dry shoot biomass; mg/cm) were measured at 28 days.

At 28 days, six blocks were used to sample the root system of one plant per pot. Loosely-adhering soil was discarded by shaking. Roots and tightly-adhering soils were frozen in liquid nitrogen, lyophilized for 48 h and then stored at -20°C. Root-adhering soil was mechanically separated (using sterile tweezers) and 0.5 g of soil was used for DNA extraction with the FastDNA SPIN kit for Soil and the FastPrep instrument (MP Biomedicals), following manufacturer's instructions. DNA was eluted in 80 µl DNase-free water and quantified using Qubit dsDNA High sensitivity Assay Kit with an Invitrogen Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) for low DNA concentrations. DNA quality was assessed using a UV spectrophotometer (NanoPhotometer NP80, Implen, Munich, Germany).

16S rRNA gene and ITS sequencing from rhizospheric DNA

A PCR reaction amplifying the V3-V4 region of the 16S rRNA gene using primers Uni341F (5′ CCTAYGGGRBGCASCAG 3′) and Uni806R (5′ GGACTACHVGGGTWTCTAAT 3′) (Yu et al., 2005; Caporaso et al., 2011; Sundberg et al., 2013) was performed in a GeneTouch Plus Thermal Cycler (Biozym Scientific, Hessisch Oldendorf, Germany). The PCR reaction contained 14.6 µl of molecular-grade water, 2.5 µl of 10 × standard reaction buffer (New England BioLabs, Ipswich, MA, USA), 1 µl of 25 mM $MgCl_2$, 1.25 µl of BSA (Bovine Serum Albumin; 2 $mg.ml^{-1}$; New England BioLabs), 2.5 µl of 2 mM dNTPs, 1 µl of each primer (0.4 µM), 0.125 µl of Hot Start DNA polymerase (5 $U.\mu l^{-1}$; New England BioLabs), and 1 µl of DNA template (5-10 $ng.\mu l^{-1}$) in a total mix of 25 µl. The PCR reaction included an initial activation step at 94°C for 2 min, followed by 30 cycles of 94°C for 20 s, 56°C for 20 s, 72°C for 40 s, and a final extension step at 72°C for 5 min. The same PCR process and conditions were used to generate libraries from the 16S rRNA gene amplicons, using primers Uni341F/Uni806R with Illumina adaptors (Nextera XT Index Kit, Illumina, San Diego, CA, USA) at Novogene (Cambridge, England), using Illumina MiSeq v.2 (2 × 250 bp) chemistry, following the manufacturer's instructions (Illumina).

The fungal ITS2 region was amplified using the primers fITS7 (5' GTGARTCATCGAATCTTTG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Gardes and Bruns, 1993; Ihrmark et al., 2012). Primers were equipped with Illumina adaptors (Nextera XT Index Kit, Illumina). To obtain high-fidelity amplification, PCR was performed using Kapa Hifi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA). The PCR was done in triplicate in a S1000 Thermal Cycler (Bio-Rad), with an initial denaturation at 95°C for 5 min, followed by 33 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 75 s and a final elongation step at 72°C for 10 min. PCR products were purified using AMPure XP beads. To assign the sequences to the respective samples, an index PCR was performed using the Illumina Nextera XT Index Kit and Kapa Hifi HotStart ReadyMix (KAPA Biosystems) according the manufacturer's instructions. PCR products were again purified with AMPure XP beads and quantified with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) following the manufacturer's instructions. For sequencing, samples were pooled, and the pools were checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Paired-end Illumina MiSeq sequencing (2 × 300 bp) was performed at the Department of Soil Ecology, UFZ-Helmholtz Centre for Environmental Research in Halle (Saale, Germany).

Sequence data processing

Amplicon sequencing datasets from 16S rRNA gene and ITS were handled independently. Sequences from the 16S rRNA gene dataset were processed and classified using the R package DADA2 (Divisive Amplicon Denoising Algorithm) v.1.12.1 pipeline (Callahan et al., 2016). Using the "FilterAndTrimmed" function, quality filtering and trimming stages were executed. Reads shorter than 100 bp were removed, allowing two errors per read. ITS sequences were processed using dadasnake v.10 (Weißbecker et al., 2020; https://github.com/a-h-b/dadasnake), with the DADA2 package (Callahan et al., 2016). Only reads with the expected amplification primers were kept, and primer sequences were cut using cutadapt v.1.18 (Martin, 2011). The amplicon reads were truncated to a minimum base quality of 7, with a minimum length of 70 nucleotides for the forward and reverse reads. For both datasets, read pairs were merged with zero mismatches, and exact sequence variants were determined as ASVs (Amplicon Sequence Variants). Chimeric reads were removed using the DADA2 "consensus" algorithm. For the 16S rRNA gene dataset, the ASVs were assigned taxonomically using the SILVA database v.138 (Quast et al., 2013), while the UNITE database v.9 was used to assign the ITS2 gene amplicon sequences taxonomically using the mothur implementation of the Bayesian Classifier (Schloss et al., 2009). During this process, any unclassified ASVs and those identified as chloroplasts, mitochondria, or eukaryotes in the 16S rRNA gene sequences were excluded from the analysis. The phyla nomenclature was maintained as suggested by the Silva database v.138 (Quast et al., 2013). The 16S rRNA gene primers have been designed to target both the archaeal and bacterial domains; hereafter, we refer to this subset of the microbiota as the prokaryotic community. For ITS, all ASVs assigned to fungi were kept. In both datasets, the rarefaction curves tended to reach a plateau, indicating that the sequencing method supplied sufficient sequences to cover most of the diversity (Figure S1). Prokaryotic and fungal taxa were identified at the genus level when possible, otherwise at family or order level.

Statistical analyses

All the data were analyzed at $P < 0.05$, using the R v.4.2.1. software (https://www.r-project.org). The relationships among soil samples based on their physicochemical composition were assessed with non-metric multidimensional scaling (NMDS) using vegan package (Oksanen et al., 2022). The data were centered and scaled, Euclidean distances were used as distance metric and two dimensions were kept for ordination with NMDS. The stress value was < 0.1. Fitting variables into NMDS plot and testing their significance was done with the envfit function.

For qPCR data, outliers were detected using the Grubbs' test (Grubbs, 1969; Burns et al., 2005) and discarded. This concerned one replicate from soil CA1, one from soil CA4, one from soil VA4, one from soil VA6 and one from soil MI2 for inoculated, non-autoclaved soils at 15 days, and one replicate from soil CA4 for inoculated, autoclaved soils at 15 days. At 15 days, in some samples, the amount of *F. graminearum* Fg1 DNA was at the quantification limit (lowest DNA concentration at which the quantification can be achieved), equal to 4.95×10^5 gene copies.g^{-1} dry soil or below. This concerned all replicates (4 in total) of soils MI3, VA5, VA2 and CA6, three replicates of soils MI2, VA4, VA7, VA1 and CA4, two replicates of CA5 and CA3, and one replicate of soils MI4, SO4 and NK4. qPCR data are presented as means ± standard errors. Firstly, these data were processed using an ANOVA, followed by Fisher's LSD tests from the *agricolae* package (de Mendiburu, 2023), to assess differences in fungistasis levels for 26 fields. Secondly, a two-way ANOVA was performed to assess the effects of field location × manure amendments. Thirdly,

differences between manured vs. non-manured fields at all 5 locations were tested with ANOVA and LSD tests. Finally, Chi^2 tests were used to assess the relationship between manure amendments and fungistasis.

The greenhouse experiment followed a randomized block design with 10 replicates (i.e., 10 pots). The data for the number of germinated seeds at 2 and 4 weeks did not display normal distribution and homogeneity of variance, based on Shapiro and Levene tests, respectively, so Kruskal-Wallis and post-hoc Dunn's tests were used to compare treatments. For shoot length, shoot biomass and density, the data displayed normal distribution and homogeneity of variance, so an ANOVA followed by Tukey's HSD tests was used. Additionally, for plant shoot length, shoot biomass and density, *t* tests were performed to compare plants grown in manured vs. non-manured soils. For shoot length, biomass and density, the plants that did not germinate were regarded as missing data (NA). In the case of shoot biomass measurements, one plant was discarded from MI2 inoculated soil and one from MI3 inoculated soil, and in the case of density, two plants were discarded from the MI2 inoculated soil and one from MI3 inoculated soil because of extreme values. Results are presented as means ± standard errors. For each plant growth parameter, letters a-c show the statistical relationship between the soils and treatments.

For the microbial communities, samples with low number of reads or ASVs were discarded. Specialized R package functions were used to determine taxa relative abundances, alpha and beta diversities and to perform statistical tests. Alpha diversity was computed, and sequences were rarefied based on the lowest number of sequences identified among samples, with a minimum of 41,961 sequences for 16S rRNA gene and 34,482 sequences for ITS. Alpha diversity indices were computed for each rarefied sample using the phyloseq (McMurdie and Holmes, 2013), microbiome (Lahti et al., 2018), or vegan (Oksanen et al., 2022) packages. Kruskal–Wallis tests were used to assess changes in alpha diversity with 10,000 permutations. If the Kruskal–Wallis test led to rejecting the null hypothesis ($P < 0.05$), LSD tests with Bonferroni correction were conducted to compare categories using *agricolae* package (de Mendiburu, 2023). Kruskal–Wallis tests were also used to assess the effect of inoculation on the relative abundance of phyla.

Beta diversity analysis was carried out using the rarefied datasets and the ASVs for both 16S rRNA gene and ITS. The dissimilarity among samples was determined by calculating the Bray-Curtis distance. The statistical significance of the comparisons was assessed using a permutation analysis of variance (PERMANOVA) with 10,000 permutations using the adonis2 function of the vegan (Oksanen et al., 2022) package. NMDS was employed to visually represent the microbial communities with the ggplot2 (Wickham, 2011) package. Analysis of similarities (ANOSIM) (with 10,000 permutations) was used to compare microbial communities of the three soils (MI2, MI4 and MI5), while pairwise comparisons were used for pairwise comparisons of microbial communities (for MI5 vs. MI4, MI5 vs. MI2, and MI4 vs. MI2).

We employed a negative binomial Wald test implemented in DESeq2 v.1.18.1 within the phyloseq package to identify taxa with significant differences to test for differential abundance (DA) on unrarefied reads (Love et al., 2014). After the Benjamini-Hochberg correction method, the taxa were considered differentially abundant when the adjusted *P* value was below 0.05. We tested the control against *Fusarium* inoculation (Fg1 samples) for each soil.

For the analysis of *Fusarium* diversity, all ASVs affiliated with the genus *Fusarium* were kept. When possible, the taxonomic identification at the species level was used, based on the UNITE database (Nilsson et al., 2019). In each soil and inoculation condition, the proportion of *Fusarium* reads among the total number of fungal reads was computed, as well as the proportion of reads for each identified *Fusarium* species among the total number of *Fusarium* reads. To

assess the impact of Fg1 inoculation on the abundance of each identified *Fusarium* species, ASV data from the eleven retrieved *Fusarium* species were treated by Kruskal-Wallis tests, followed by post-hoc LSD tests with Benjamini-Hochberg correction ($P < 0.05$).

RESULTS

Soil fungistasis of Serbian fields against *Fusarium graminearum*

Before soil inoculation (day 0), *F. graminearum* Fg1 was not found in any of the 26 soils analyzed, implying that any observed Fg1 DNA increase or decrease over the 15 days of experiment was specifically due to Fg1 growth or decline after inoculation. When autoclaved soils were used, growth of *F. graminearum* Fg1 took place in all soils during the 15 days of soil incubation, to a magnitude of 2 $\log_{10}$ units or more (Figure 2A). When non-autoclaved soils were used, levels of *F. graminearum* Fg1 were always lower than with autoclaved soils. The pathogen was stable or even grew in 16 of 26 non-autoclaved soils but, interestingly, the amount of Fg1 DNA decreased in the 10 others (i.e., 38%; all from western/central Serbia), indicating a fungistasis potential.

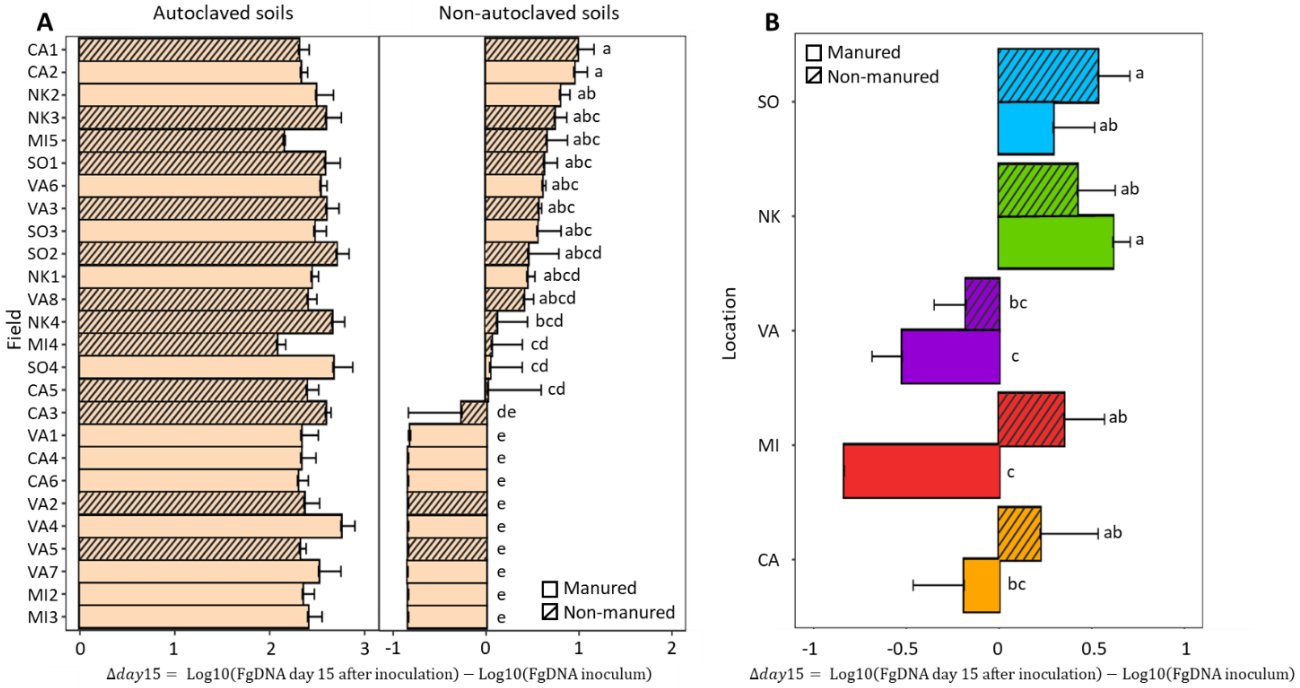

Figure 2. Amount of *Fusarium graminearum* Fg1 DNA present in the soils at 15 days after inoculation. (A) DNA quantity of *Fusarium graminearum* Fg1 in inoculated 26 Serbian soils, from Sombor (SO), Novi Karlovci (NK), Valjevo (VA), Mionica (MI) and Čačak (CA), in autoclaved and non-autoclaved soils, after 15 days of incubation under controlled conditions. Results are presented as means with standard errors. Striped bars indicate soils without manure amendments and non-striped bars indicate soils with manure amendments. Two-way ANOVA ($P < 0.05$) showed that field location and manure amendments were significant factors, but the interaction between them was not significant. Differences between individual soils were assessed with ANOVA and LSD tests ($P < 0.05$; letters a-e are used to show statistical differences). (B) Comparison of manured vs. non manured soils at each location. Results are presented as means with standard errors. Striped bars are used for soils without manure amendments and non-

striped bars for soils with manure amendments. Differences between manured vs. non-manured soils at all locations were tested with ANOVA and LSD tests ($P < 0.05$; letters a-c are used to show statistical differences).

Two-way ANOVA ($P < 0.05$) showed that field location and manure amendments were significant factors, but the interaction between them was not significant. When considering manure amendments, 7 of 10 non-autoclaved soils (70%) displaying fungistasis had been amended, whereas only 6 of 16 non-autoclaved, non-fungistatic soils (37%) had received manure (Figure 2A). When locations were compared (Figure 2A), fungistasis was found for the three western/central Serbia locations (Valjevo, Mionica and Čačak) for manure-amended soils, but only for Valjevo and Čačak for non-manured soils. Relationship between manure amendments and fungistasis was not significant at $P < 0.05$ ($Chi^2 = 1.463$), but Fg1 growth was significantly lower in manured soils than in non-manured soils from Mionica (LSD test, $P < 0.01$), with a similar trend (although not significant) in Čačak soils (Figure 2B). In summary, fungistasis was observed for 38% of the 26 soils, and manure amendment was identified as a significant factor determining fungistasis at some (especially Mionica) but not all geographic locations.

Relation between soil composition and fungistasis

NMDS of soil physicochemical data showed that soils mainly clustered according to their geographical location (permutation test, $P < 0.001$), which coincided also with particular soil types, whereas manure amendment did not have an over-riding effect overall (Figure 1B). However, higher organic matter content (OM) was found in fungistatic (and manured) soils MI2 (7.66%) and MI3 (6.96%), compared with non-fungistatic (and non-manured) soils MI4 (5.87%) and MI5 (5.88%) (Table S2). Higher potassium (K) content was also evidenced in fungistatic MI2 (370 mg/kg) and MI3 (293 mg/kg) than in non-fungistatic soils MI4 (218 mg/kg) and MI5 (184 mg/kg). When comparing fungistatic and non-fungistatic soils at other locations (Valjevo or Čačak), fungistatic soils did not display higher contents in organic matter or potassium compared with non-fungistatic soils, and they did not exhibit any other chemical particularity. In summary, soils exhibited particularities according to the location of origin (and soil type), and in Mionica according to fungistasis status (confounded with manure usage; with higher OM and K contents). Therefore, there was no global relation between soil composition and fungistasis.

Suppressiveness of soils from Mionica against *Fusarium graminearum*-induced wheat damping-off

Based on the contrasted fungistasis results and the link with manure amendments found in Mionica, we selected these soils for a wheat damping-off suppressiveness assay with *F. graminearum* Fg1. At 14 days after sowing, the number of germinated seeds was statistically lower upon pathogen inoculation in soil MI4, whereas the difference was not significant in soils MI2, MI3, and MI5 (Figure 3A). Similarly, at 4 weeks, the number of plants alive was statistically lower in *F. graminearum* Fg1-inoculated vs. non-inoculated MI4 soils, while this difference was not significant in the three other soils (Figure 3B). Inoculation with *F. graminearum* Fg1 did not significantly impact wheat shoot length (Figure S2A), but it resulted in lower dry shoot biomass (Figure S2B) and shoot density (Figure S2C) in soil MI2. In addition, dry shoot biomass, shoot length and shoot density were higher overall (*t* tests, all $P < 10^{12}$) in manured soils (MI2 and MI3) than in non-manured soils (MI4 and MI5). In summary, non-fungistatic (non-manured) soil MI4

was also conducive to wheat damping-off caused by *F. graminearum* Fg1 but non-fungistatic (non-manured) soil MI5 was suppressive, whereas fungistatic (manured) soils MI2 and MI3 were suppressive based on wheat germination and survival (even though plant growth in MI2 was affected by the pathogen).

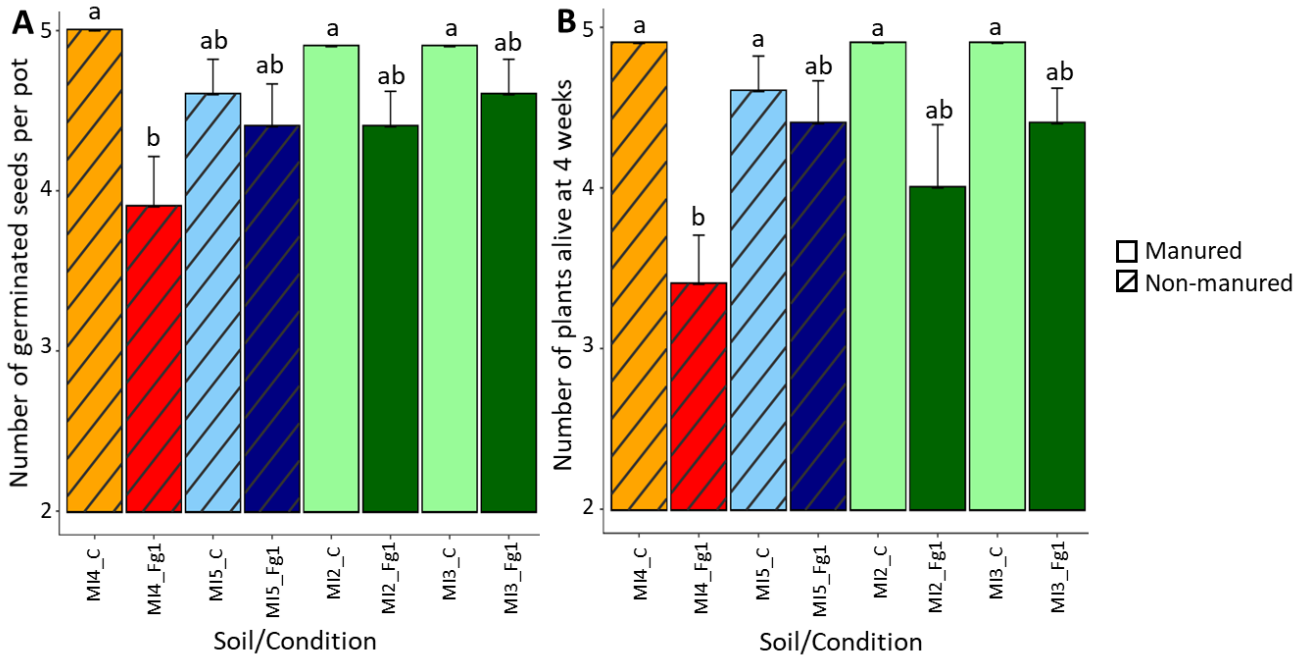

Figure 3. Wheat suppressiveness assay with soils from Mionica (MI4, MI5, MI2 and MI3), non-inoculated (shown as MIi_C) or inoculated with *Fusarium graminearum* Fg1 (shown as MIi_Fg1). Soils that did not receive manure amendments are represented with stripes. All results are presented as means and standard errors (n = 10). Data were treated with Kruskal-Wallis and Dunn's tests ($P < 0.05$). For each soil, statistical differences are shown with letters a and b. (A) Number of germinated wheat seeds per pot (out of 5) at 2 weeks. (B) Number of wheat plants alive per pot at 4 weeks.

Diversity and genetic structure of prokaryotic and fungal rhizospheric communities

When assessing the link between rhizosphere microbial diversity and disease-suppressiveness status of soils from Mionica, metabarcoding data for the 16S rRNA gene (prokaryotic community) pointed to similar diversity levels for the three soils (Figure 4ABC). This was found whether soils were inoculated or not, except that Pielou index (evenness) in the fungistatic, suppressive soil MI2 (also the only manured soil) was significantly higher than in non-fungistatic soils MI4 (conducive) and MI5 (suppressive) when inoculated with Fg1 (Figure 4C). Besides that, the effect of Fg1 inoculation on alpha diversity was not significant, regardless of the soil and the diversity index. With ITS metabarcoding data (fungal community) from the rhizosphere, the Shannon (diversity; Figure 4D) and Pielou (evenness; Figure 4F) indices but not the Chao1 index (richness; Figure 4E) were statistically higher (i) in soils MI4 (non-fungistatic, non-suppressive) and MI2 (fungistatic, suppressive) than in MI5 (non-fungistatic, suppressive) in the absence of inoculation, and (ii) in soil MI4 than in MI5 when Fg1 had been inoculated. Inoculation itself resulted only in a lower Pielou index in soil MI4 (Figure 4F).

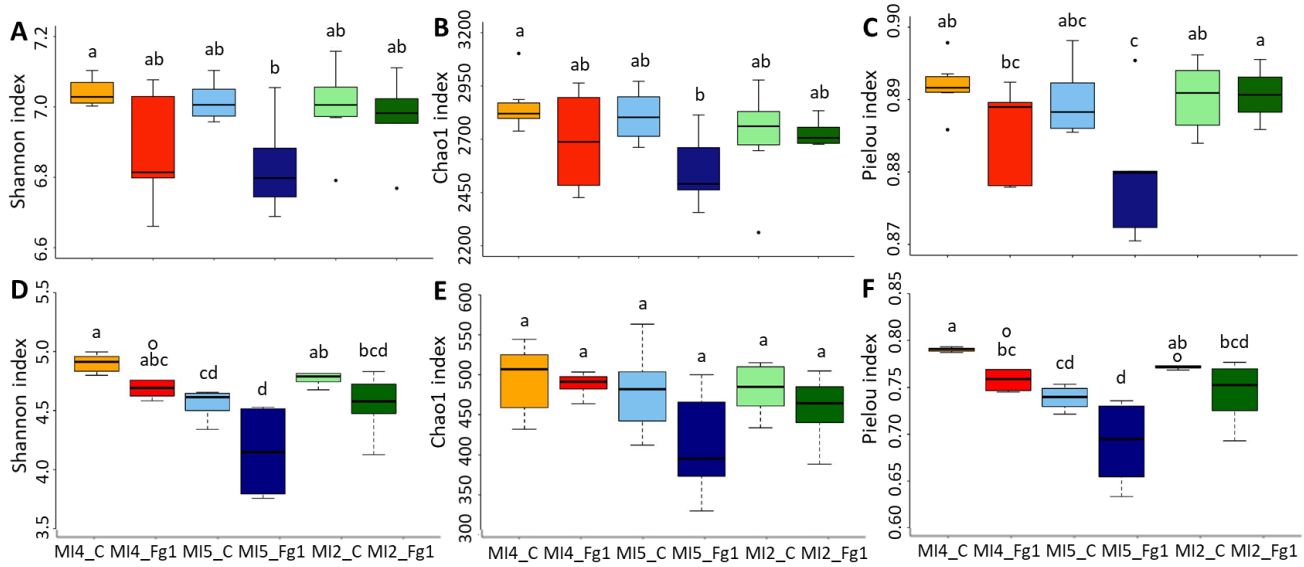

Figure 4. Alpha diversity of prokaryotic (A, B, C) and fungal (D, E, F) rhizosphere communities in soils from Mionica (MI4, MI5 and MI2) inoculated with *Fusarium graminearum* Fg1 (shown as MIi_Fg1) or non-inoculated (shown as MIi_C). Data were compared using Kruskal–Wallis tests, followed by Fisher's Least Significant Difference (LSD) tests with Bonferroni correction. Letters a-d indicate statistical relations ($P < 0.05$) between soils × inoculation (Fg1 or not) combinations.

NMDS plots based on Bray-Curtis distances showed that microbial communities clustered largely according to the location of origin, for the prokaryotic (Figure 5A) and especially the fungal community (Figure 5B). Indeed, individual soils accounted for 42.6% (for prokaryotes) and 60.0% (for fungi) of the variations in community structure (PERMANOVA, both at $P < 0.001$), whereas merely 3.7% (prokaryotes; $P = 0.048$) and 4.0% (fungi; $P = 0.023$) of the differences were attributed to inoculation (Table S3). When considering each soil separately, the effect of Fg1 inoculation was significant in most cases, i.e., for MI2 ($P = 0.009$ for prokaryotes and $P = 0.004$ for fungi), MI5 ($P = 0.004$ for prokaryotes and $P = 0.048$ for fungi), and MI4 ($P = 0.004$ for prokaryotes but $P > 0.05$ for fungi).

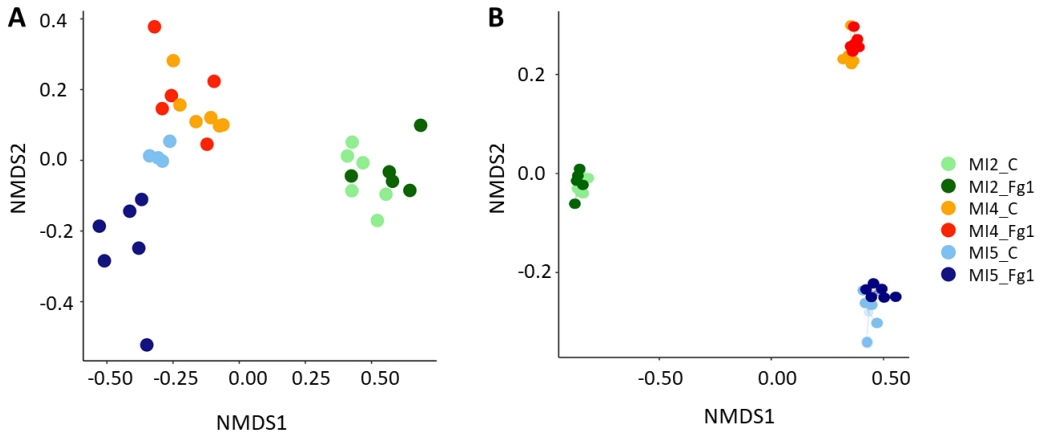

Figure 5. Non-Metric Multidimensional Scaling (NMDS) of soils MI4, MI5 and MI2 inoculated with *Fusarium graminearum* Fg1 (shown as MIi_Fg1) or non-inoculated (shown as MIi_C) based on rhizosphere metabarcoding of prokaryotic (A) and fungal (B) communities. ANOSIM (10,000

permutations) indicated that the between-groups difference was larger than the within-groups difference ($P = 10^{-4}$ for prokaryotes and 10^{-4} for fungi). All pairwise comparisons (for MI5 vs. MI4, MI5 vs. MI2, and MI4 vs. MI2) for prokaryotes were $P = 10^{-3}$ and $P = 10^{-3}$ for fungi.

In summary, most differences in prokaryotic alpha diversity were not significant, whereas fungi in soil MI5 (non-fungistatic, suppressive) displayed lower Shannon and Pielou indices. In addition, microbial community structure depended mostly on the location of origin, with a modest but significant effect of inoculation.

Composition of the prokaryotic rhizosphere community

The most abundant rhizosphere phyla in soils MI2, MI4 and MI5 were the same, i.e., *Proteobacteria*, *Actinobacteriota, Firmicutes, Chloroflexi, Verrucomicrobiota* and *Crenarchaeota.* The 20 most abundant taxa (the lowest likely taxonomic information available for an ASV, often at the genus level) in the prokaryotic community represented an average of 56.2 % (MI4 = 55.3%, MI5 = 58.6%, MI2 = 54.2%) of the sequences (Figure 6ABC). Some of these most abundant taxa were evidenced in all three soils, e.g., the *Actinobacteriota Gaiella* (average 4.6% of the reads) and a taxon affiliated to the order *Gaiellales* (average 13.0%). Some were found in specific soil(s), as for (i) the *Proteobacteria* genus *Sphingomonas* in soil MI4 (1.4%), (ii) an *Elsterales* (*Proteobacteria*) genus in soil MI5 (1.0%), (iii) an *Acidobacteriota* taxon from the order *Vicinamibacterales* (1.1%) and various *Actinobacteriota*, i.e., the genera *Microlunatus* (2.1%) and *Rubrobacter* (1.6%), a *Microtrichales* genus (1.3%) and a *Ilumatobacteraceae* genus (1.1%), which were evidenced only in soil MI2, and (iv) the *Actinobacteriota* genera *Conexibacter* (2.0%), *Marmoricola* (1.0%), *Intransporangium* (1.3-1.1%) and *Acidothermus* (2.0-1.9%) in soils MI4 and MI5.

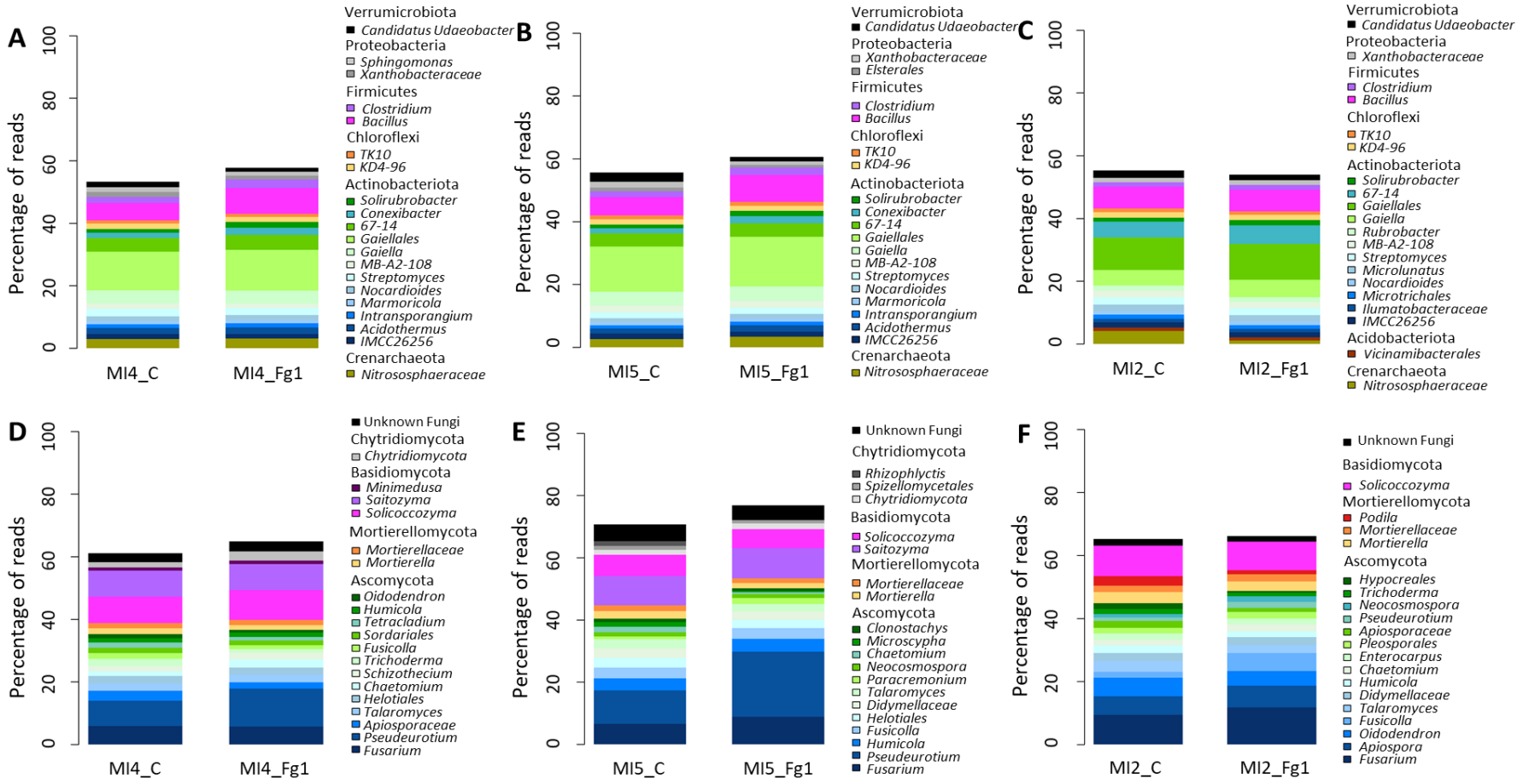

Figure 6. Top 20 most abundant prokaryotic (A, B, C) and fungal taxa (D, E, F) in the wheat rhizosphere of soils from Mionica MI4, MI5 and MI2. MI_C, control (non-inoculated soils); MIi_Fg1, *Fusarium graminearum*-inoculated soils. The 20 most abundant taxa (the lowest taxonomic information available for an ASV; often at genus level) in the prokaryotic community represented 53.2% (non-inoculated MI4 soil), 57.7% (Fg1-inoculated MI4 soil), 55.7% (non-inoculated MI5 soil), 60.5% (Fg1-inoculated MI5 soil), 55.3% (non-inoculated MI2 soil) and 53.9% (Fg1-inoculated MI2 soil) of the sequences, whereas the 20 most abundant fungal taxa represented 65.2% (non-inoculated MI2 soil), 66.1% (Fg1-inoculated MI2 soil), 61.1% (non-inoculated MI4 soil), 64.8% (Fg1-inoculated MI4 soil), 70.7% (non-inoculated MI5 soil) and 76.9% (Fg1-inoculated MI5 soil) of the sequences.

Inoculation with *F. graminearum* Fg1 resulted in a significant increase ($P < 0.05$) in the rhizosphere relative abundance of the phylum *Firmicutes* in the non-fungistatic soils MI4 (from 10.8% to 15.8%) and MI5 (from 10.1% to 14.4%) (Figure S3). In the fungistatic MI2 soil, pathogen inoculation caused a modest but significant increase ($P < 0.05$) in the relative abundance of *Actinobacteriota* (from 48.0% to 50.9%) and *Proteobacteria* (from 13.7% to 17.1%), but led to somewhat lower levels of *Crenarchaeota* (from 5.4% to 1.5%) and *Chloroflexi* (from 5.4% to 4.9%) ($P < 0.05$).

Differential analysis was also used to identify individual taxa that differed significantly ($P < 0.05$) in relative abundance between *F. graminearum* Fg1-inoculated and non-inoculated samples, at the scale of the whole rhizosphere community. Among the 1493 identified prokaryotic taxa (Figure 7), this concerned 17 taxa in soil MI4 (non-fungistatic, non-suppressive), 45 taxa in soil MI5 (non-fungistatic, suppressive), and 17 taxa in soil MI2 (fungistatic, suppressive). Most of the taxa were found exclusively in one of the three soils, but *Gemmatimonas* (*Gemmatimonadota*) was evidenced in all three soils, with a lower abundance in inoculated than in non-inoculated samples (by 0.8, 0.5 and 1.2 $\log_2$ units for soils MI2, MI4 and MI5, respectively). In both soils MI4 and MI5, a taxon belonging to the candidate group SC-I-84 (*Proteobacteria*) was found more in non-inoculated than in inoculated samples (by 0.8 and 1.2 $\log_2$ units, respectively), as for a *Myxococcota* taxon from the candidate group Blrii41 (by 0.6 and 1.2 $\log_2$ units, respectively), whereas the opposite was found for *Bacillus* (by 0.7 and 0.9 $\log_2$ units, respectively), *Paenibacillus* (by 0.8 and 0.9 $\log_2$ units, respectively) and *Pelosinus* (*Firmicutes*) (by 1.2 and 2.0 $\log_2$ units, respectively). *Sphingobium* (*Proteobacteria*) was more abundant in inoculated than in non-inoculated samples of soils MI2 and MI5 (by 7.0 and 8.0 $\log_2$ units, respectively). These inoculation effects concerned also some of the 20 most abundant prokaryotic taxa, i.e., for *Solirubrobacter* in soil MI4, *Candidatus Udaeobacter* and *Bacillus* for soil MI5 and for *Nitrososphaeraceae* in soil MI2.

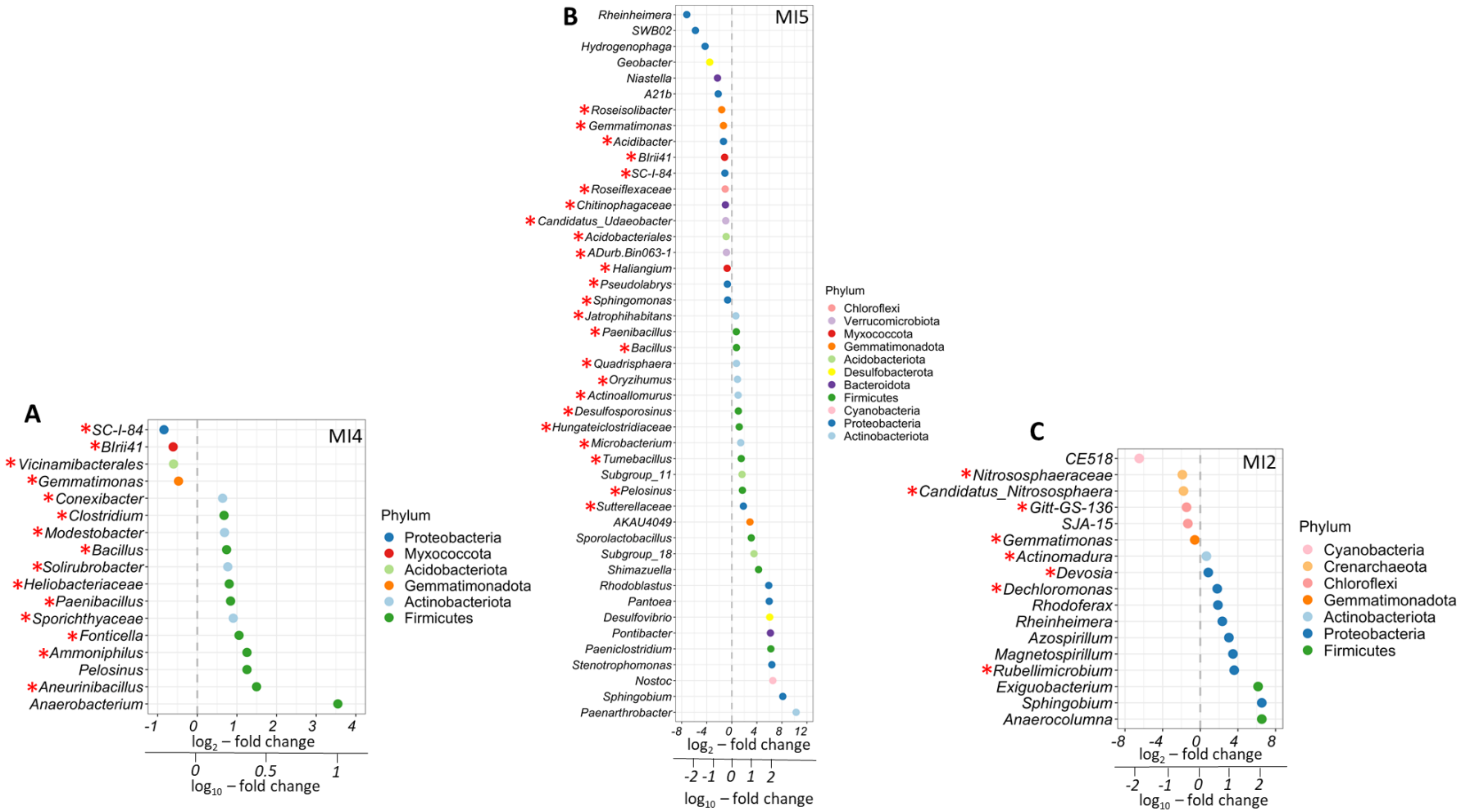

Figure 7. Differential abundance analysis of prokaryotic taxa in the wheat rhizosphere of soils MI4 (A), MI5 (B) and MI2 (C), following inoculation with *Fusarium graminearum* Fg1. The X axes are shown with $\log_2$- and $\log_{10}$-fold changes. Negative log changes (significantly more abundant in non-inoculated soils); positive log changes (significantly more abundant in *Fusarium graminearum* Fg1-inoculated soils). All taxa shown were affected by inoculation ($P < 0.05$), and those representing more than 0.1 % of all sequences are indicated with an asterisk.

In summary, the wheat rhizosphere of the three soils shared the main phyla and the majority of the most abundant taxa, yet several taxa were soil specific. Additionally, soil inoculation with *F. graminearum* Fg1 impacted the rhizosphere microbial community, but often with soil-specific effects.

Composition of the fungal rhizosphere community

In each soil, *Ascomycota*, *Basidiomycota*, and *Mortierellomycota* were the phyla harboring the most abundant taxa. However, differences were found between soils, as in soils MI4 and MI5 the phylum *Chytridiomycota* was also present. The 20 most abundant fungal taxa (considered at the genus level or higher rank if information not available) represented 61% (in non-inoculated MI4 soil) to 77% (in Fg1-inoculated MI5 soil) of the reads in rhizosphere samples (Figure 6DEF). Distinctive features were evidenced in particular soil(s), as (i) *Schizothecium*, *Sordariales*, *Tetracladium*, *Minimedusa* were found only in soil MI4, (ii) *Clonostachys*, *Microscypha*, *Paracremonium* only in soil MI5, (iii) *Podila* (a *Mortierellaceae* genus; representing 10% of the reads), *Hypocreales*, *Apiospora*, *Pleosporales, Enterocarpus* were found only in soil MI2, (iv) *Pseudeurotium, Helotiales, Humicola*, *Saitozyma* only in soils MI4 and MI5, (v) *Apiosporaceae*, *Chaetomium*, *Trichoderma*, *Oidodendron* only in soils MI4 and MI2, and (vi) *Neocosmospora*, *Didymellaceae* only in soils MI5 and MI2.

At phylum level, inoculation with *F. graminearum* Fg1 resulted into a significant increase ($P < 0.05$) in the rhizosphere relative abundance of the *Chytridiomycota* in the non-fungistatic soil MI4 (from 5.4% to 7.8%), and a decrease of the *Mortierellomycota* (from 8.7% to 6.6%) in the fungistatic soil MI2 (Figure S3).

When differential analysis was performed to assess inoculation effects at the scale of the whole fungal community (Figure 8), decreased levels were found in *F. graminearum* Fg1-inoculated soils for: (i) *Ascomycota* genera *Beauvaria* (by 5.8 $\log_2$ units) and *Collarina* (by 4.9 $\log_2$ units) and *Mortierellomycota* genus *Podila* (by 1.8 $\log_2$ units) in soil MI4 (non-fungistatic, non-suppressive), for (ii) four genera (of distinct phyla) including *Waitea* (*Basidiomycota*; by 24 $\log_2$ units), *Microscypha* (*Ascomycota*; by 2 $\log_2$ units), *Paraglomus* (*Glomeromycota*; by 7 $\log_2$ units) and *Rhizophlyctis* (*Chytridiomycota*; by 4 $\log_2$ units) in soil MI5 (non-fungistatic, suppressive), and (iii) the four *Ascomycota* genera *Septoria* (by 23 $\log_2$ units), *Purpureocillium* (by 2 $\log_2$ units), *Scedosporium* (by 6 $\log_2$ units) and *Exophiala* (by 2.5 $\log_2$ units) in soil MI2 (fungistatic, suppressive). Significantly higher levels were found in *F. graminearum* Fg-inoculated MI2 soils for *Atractium* (by 2.5 $\log_2$ units) and *Scutellinia* (by 3.0 $\log_2$ units). Inoculation effects were also observed (Kruskal-Wallis tests and Fisher's tests with Bonferroni correction) for some of the 20 most abundant fungal taxa, but these effects were not significant anymore with the more stringent differential analysis.

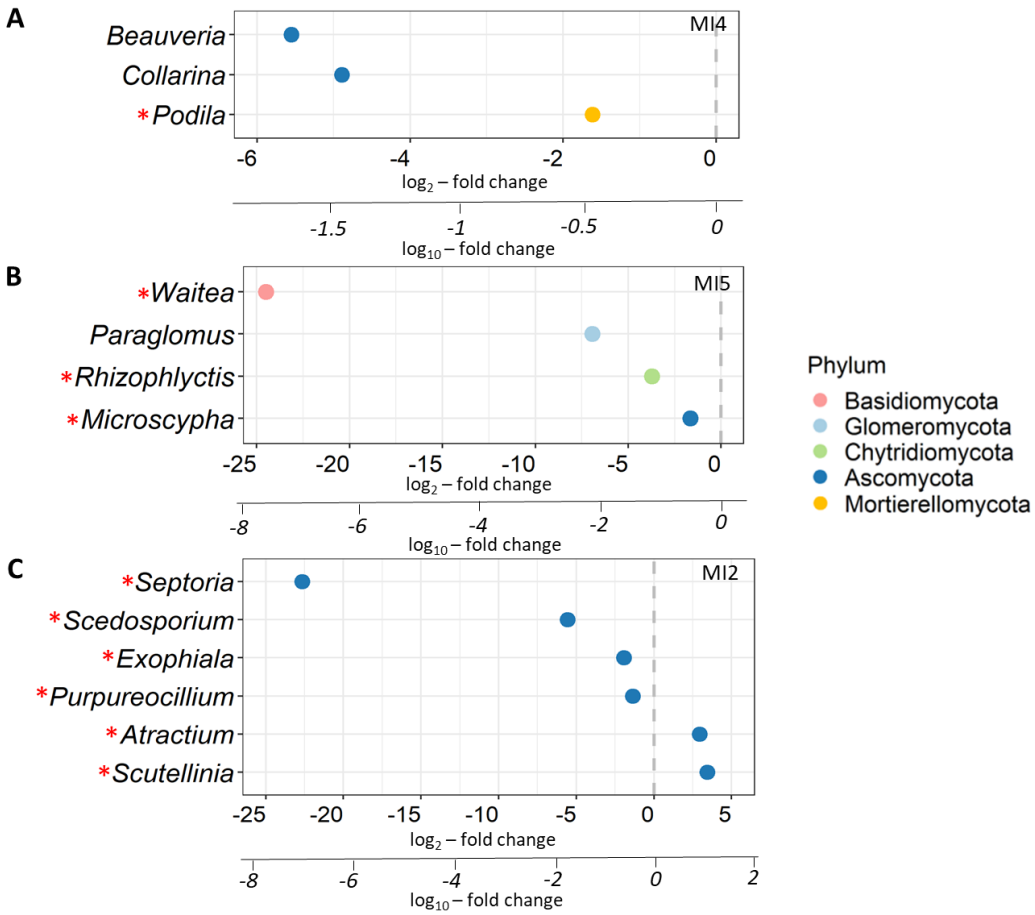

Figure 8. Differential abundance analysis of fungal taxa in the wheat rhizosphere of soils MI4 (A), MI5 (B) and MI2 (C) following inoculation with *Fusarium graminearum* Fg1. The X axes are shown with $\log_2$- and $\log_{10}$-fold changes. Negative log changes (significantly more abundant in non-inoculated soils); positive log changes (significantly more abundant in *Fusarium graminearum* Fg1-inoculated soils). All taxa shown were affected by inoculation ($P < 0.05$), and those representing more than 0.1 % of all sequences are indicated with an asterisk.

In summary, the three soils harbored representatives from the phyla *Ascomycota*, *Basidiomycota*, and *Mortierellomycota*, while taxa from the order *Chytridiomycota* were found only in soils MI4 and MI5. Similarly to the prokaryotic community, soil inoculation with *F. graminearum* Fg1 impacted fungal rhizosphere community.

Composition of the *Fusarium* community

In the absence of *F. graminearum* Fg1 inoculation, the *Fusarium* genus represented 9.4% of all rhizosphere fungi in non-suppressive soil MI4 (also non-fungistatic), vs. only 5.9% and 6.6% in suppressive soils MI5 (non-fungistatic) and MI2 (fungistatic), respectively (Table S4). The *Fusarium* genus was more prevalent in Fg1-inoculated vs. non-inoculated rhizosphere for soil MI4 (up to 11.8%) and MI2 (up to 8.8%), but not for soil MI5. Within the genus, inoculation increased ($P < 0.05$) rhizosphere levels of *F. graminearum* from 2.6% to 25.0% of all *Fusarium* sequences for MI4, 0.9% to 29.0% for MI5, and 0.5% to 20.0% for MI2 (Figure 9). In addition, a small decrease in levels of *F. equiseti* was found in soil MI4 after inoculation (Table S3).

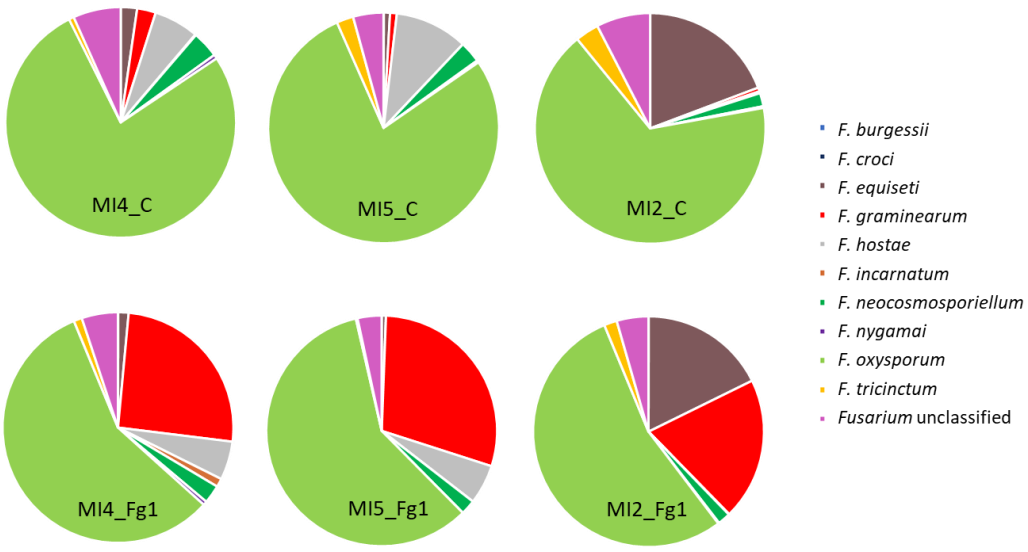

Figure 9. Proportion of the different *Fusarium* species in the wheat rhizosphere of soils MI4, MI5 and MI2 inoculated (MIi_Fg1) or not (MIi_C) with *Fusarium graminearum* Fg1.

DISCUSSION

Soils are known to regulate growth or sporulation of phytopathogens, potentially inhibiting the establishment of a new infectious cycle (Alabouvette, 1986; Garbeva et al., 2011; Cha et al., 2016; Legrand et al., 2019; Tao et al., 2020). In certain cases, this can affect survival of fungal pathogens, which corresponds to fungistasis (Lockwood, 1977; Garbeva et al., 2011; Legrand et al., 2019). Fungistasis is an important soil trait, but it remains insufficiently understood.

In the current work, we tested the hypotheses that soil fungistasis can be promoted by manuring, a standard farming practice in regions with livestock farming. Screening of 26 Serbian soils evidenced 10 soils in which the pathogen *F. graminearum* declined significantly. The infectious cycle of *F. graminearum* includes a phase where the pathogen must survive in the soil before infecting new seedlings (Pereyra et al., 2004; Cobo Díaz et al., 2019). This decline was due to antifungal properties of the soil microbiota, as *F. graminearum* grew readily when the 10 soils were sterilized, to the same extent as in the non-fungistatic soils. All natural soils can exert some level of pathogen control due to the presence of an active microbiota (Lockwood, 1977), but without necessarily achieving fungistasis.

In this study, fungistasis was only found in Valjevo (eutric cambisols and pseudogleys), Čačak (vertisols) and Mionica (vertisols) soils of the hilly region in western/central Serbia, where agriculture is less intensive and follows more traditional practices than in northern plains of Serbia, where soils also differ (chernozems). Microbial communities may vary with soil type and management (Karimi et al., 2020), whereas farming practices e.g., tillage can affect the survival of phytopathogens in soil (Legrand et al., 2017; Supronienė et al., 2023). Control of fungal hyphal growth by the soil microbiota may be influenced by the physicochemical properties of the soil and soil management practices (Zhang et al., 2020; Bellini et al., 2023). Here, a majority of fungistatic soils and a minority of non-fungistatic soils were manured soils. In Mionica, only manured soils were fungistatic, and they differed statistically from non-manured soils based on fungistasis. Animal manure amendment brings new microorganisms, supplies nutrients to the

soils and impacts the resident microbiota (Mousa and Raizada, 2016; Su et al., 2022), but this was not sufficient to develop fungistasis in the chernozems of northern Serbia.

The positive effect of manure on fungistasis may also materialize by different soil physicochemical properties, as the latter can influence microbiota functioning including phytoprotection properties (Sipilä et al., 2012; Almario et al., 2014). Soil fungistasis to *F. graminearum* was related to manganese and nitrogen contents in Brittany soils (Legrand et al., 2019), but here soil types were more diverse and physicochemical properties varied primarily with geographic location (and soil type). In Mionica, where the relation between manure and fungistasis was significant, the manured, fungistatic soils displayed higher organic matter and potassium contents, which may be due to manuring itself (Aziz et al., 2010). The addition of potassium phosphite enriched the community in antagonistic bacteria and affected survival of the tomato pathogen *Ralstonia solanacearum* in soils (Su et al., 2022).

Using soils from Mionica, we tested whether fungistatic soils (manured) would also be suppressive to disease. This possibility has been mentioned in earlier work (Lockwood, 1977; Garbeva et al., 2011). Here, the two manured soils from Mionica, MI2 and MI3 were suppressive to *F. graminearum* disease of wheat, and it could be that fungistasis promoted rhizosphere-based disease-suppressiveness. However, one non-manured soil was non-fungistatic and disease-conducive (MI4), but the other non-manured, non-fungistatic soil (MI5) was suppressive. This raises the possibility that manuring was not necessarily a primary determinant for biocontrol microorganisms in the rhizosphere of soils from Mionica. When sampling was done for the fungistasis assay in October 2020, fields were grown with alfalfa (MI2), sunflower (MI3), wheat (MI4) or a meadow (MI5), while when sampling was done for suppressiveness assay in June 2021, fields were grown with maize (MI2), wheat (MI3 and MI5) or a meadow (MI4) (Table S1), and perhaps this contributed to the difference between MI4 and MI5. A wide range of bacterial and fungal taxa may be involved in disease suppression (Kloepper et al., 1980; Tamietti and Alabouvette, 1986; Weller et al., 2002; Ossowicki et al., 2020; Yadav et al., 2021). In the present study, the comparison of the top 20 prokaryotic and fungal taxa did not allow the distinction of taxa more abundant in the two disease-suppressive soils MI2 and MI5, compared with conducive soil MI4, probably because phytoprotection entailed the joint contribution of various microbial taxa (Alabouvette et al., 1985; Rouxel and Sedra, 1989; Rasmussen et al., 2002; Kyselková et al., 2009; Ossowicki et al., 2020). When *F. graminearum* was added, Pielou index (evenness) for bacteria was higher in manured MI2 soil than non-manured soils MI4 and MI5, in accordance with higher bacterial diversity found in soils amended with manure (Fu et al., 2017).

In fungistatic soil MI2, *F. graminearum* was evidenced in the rhizosphere albeit at a lower relative abundance than in the other soils from Mionica (Figure 9), but in the second suppressive soil MI5, *F. graminearum* remained at higher levels within the *Fusarium* genus, pointing to the importance of rhizosphere interactions for wheat protection in soil MI5. Control of pathogen infection was described in tobacco exposed to the black root rot pathogen *Thielaviopsis basicola*, and was attributed to stimulation of plant defenses (Almario et al., 2014), as in carnation (Van Peer et al., 1991), tomato (Tamietti et al., 1993) or radish (Leeman et al., 1995) confronted to the Fusarium wilt pathogen *F. oxysporum*.

In conclusion, we identified manure as a key farming practice for soil fungistasis towards the wheat pathogen *F. graminearum*. At the Mionica location where manure is of particular importance for fungistasis, the two fungistatic soils but also one of the non-fungistatic soils were suppressive to *F. graminearum* disease in wheat, and suppressiveness was related to particularities in rhizosphere microbial diversity.

SEQUENCE ACCESSION NUMBERS

For 16S rRNA gene and ITS, the raw amplicon data were deposited at the NCBI Sequence Read Archive (SRA) under the BioProject PRJNA1010537.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICAL STATEMENT

The experiments did not involve human participants and/or animals.

SUPPLEMENTARY DATA

Table S1. Locations, sample ID, type and quantity (t/ha) of manure, recent cropping field history, use of fertilizer and pesticides, postharvest residues management and the observed presence of wheat fusariosis of 26 Serbian soils.

Location	Sample ID	Soil type*	Type of animal manure	Manure quantity	Recent cropping history (most recent in bold)**	Use of mineral fertilizers	Use of pesticides	Postharvest residues management	Observed presence of wheat fusariosis in recent years
Sombor	SO1	Chernozem	-	-	**Wheat**-soybean-maize	Yes	Yes	Ploughing	Yes
	SO2	Chernozem	-	-	**Wheat**-soybean-maize	Yes	Yes	Ploughing	Yes
	SO3	Chernozem	Beef	35 t/ha	**Wheat**-maize-wheat-maize	Yes	Yes	Ploughing	Yes
	SO4	Chernozem	Beef	35 t/ha	**Wheat**-maize-wheat-maize	Yes	Yes	Ploughing	Yes
Novi Karlovci	NK1	Chernozem	Beef	14.5 t/ha	**Wheat**-maize-sunflower-beetroot	Yes	Yes	Ploughing	No
	NK2	Chernozem	Beef	14.5 t/ha	**Wheat**-maize-sunflower-beetroot	Yes	Yes	Ploughing	No
	NK3	Chernozem	-	-	**Wheat**-sunflower-beetroot-maize-beetroot	Yes	Yes	Ploughing	No
	NK4	Chernozem	-	-	**Wheat**-sunflower-beetroot-maize-beetroot	Yes	Yes	Ploughing	No
Valjevo	VA1	Eutric cambisol	Sheep, beef and chicken	70-80 t/ha	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	No
	VA2	Eutric cambisol	-	-	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Burning	No
	VA3	Eutric cambisol	-	-	**Oat**-wheat-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	No
	VA4	Eutric cambisol	Sheep, beef and chicken	70-80 t/ha	**Maize**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	No
	VA5	Pseudogley	-	-	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing and burning	No

	VA6	Pseudogley	Beef	80 t/ha	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing and burning	No
	VA7	Pseudogley	Beef	80 t/ha	**Maize**-wheat-maize-wheat-maize	Yes	Yes***	Ploughing	No
	VA8	Pseudogley	-	-	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	No
Mionica	MI2	Vertisol	Sheep	80 t/ha	**Alfalfa**-wheat-maize-wheat-maize	Yes	Yes***	Ploughing	No
	MI3	Vertisol	Sheep	80 t/ha	**Sunflower**-wheat-maize-wheat-maize	Yes	Yes***	Ploughing	No
	MI4	Vertisol	-	-	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	No
	MI5	Vertisol	-	-	**Meadow**-wheat-maize-wheat-maize	Yes	Yes***	Ploughing	No
Čačak	CA1	Vertisol	-	-	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	Yes
	CA2	Vertisol	Beef	30-40 t/ha	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	No
	CA3	Vertisol	-	-	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	Yes
	CA4	Vertisol	Beef	30-40 t/ha	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	No
	CA5	Vertisol	-	-	**Wheat**-maize-wheat-maize-wheat	Yes	Yes***	Ploughing	Yes
	CA6	Vertisol	Beef	30-40 t/ha	**Maize**-wheat-maize-wheat-maize	Yes	Yes***	Ploughing	No

* Soil type was determined according to pedological maps by Tanasijević et al. (1964) and Nejgebauer et al. (1971)
** Soil for fungistasis tests was taken in autumn 2020 (no crop present), and soil for plant tests was taken in spring 2021 at Mionica (maize in MI2, wheat in MI3 and MI5, meadow in MI4).
*** Only herbicides used, no fungicides

Table S2. Physicochemical properties of the 26 Serbian soils.

Soil	Sand (%)	Silt (%)	Clay (%)	CEC (cmol/kg)	CEC_sat (%)	pH	Humus (%)	OM (%)	N_tot (%)	C:N	P_tot (mg/kg)	K_tot (mg/kg)	Fe_tot (%)
CA1	27.9	32.9	39.2	29.0	66	5.70	3.33	5.79	0.17	19.8	15.7	168.5	2.88
CA2	26.4	23.8	49.8	34.1	77	6.23	2.65	4.91	0.13	22.0	7.9	214.2	3.31
CA3	25.0	31.3	43.7	29.2	88	6.99	4.16	5.38	0.21	14.9	16.7	268.2	3.05
CA4	21.1	31.0	47.9	33.6	72	5.94	3.54	6.56	0.18	21.2	5.0	283.1	3.33
CA5	20.2	31.7	48.1	35.4	71	6.22	3.80	6.37	0.19	19.5	17.7	224.2	3.30
CA6	26.2	29.8	44.0	32.7	68	5.96	3.51	5.99	0.18	19.3	17.5	226.6	3.42
MI2	18.1	22.9	59.0	48.8	98	7.62	3.89	7.66	0.19	23.4	47.9	370.3	3.99
MI3	17.9	28.0	54.1	45.1	98	7.81	4.27	6.96	0.21	19.3	26.1	293.1	3.57
MI4	17.7	31.3	51.0	33.0	75	6.00	2.98	5.87	0.15	22.8	12.6	218.3	3.97
MI5	24.5	27.1	48.4	33.0	75	6.35	3.77	5.88	0.19	18.0	15.1	183.5	3.66
NK1	37.0	37.7	25.3	48.2	99	8.00	4.83	6.97	0.24	16.9	75.3	188.5	2.57
NK2	37.3	37.2	25.5	48.1	99	8.07	4.42	6.81	0.22	18.0	66.4	146.1	2.74
NK3	33.3	37.4	29.3	49.1	99	7.82	4.92	6.75	0.25	15.7	101.6	187.6	2.60
NK4	34.5	39.8	25.7	48.7	99	8.08	4.39	6.87	0.22	18.2	88.8	174.3	2.48
SO1	49.1	28.6	22.3	49.2	99	7.94	4.12	5.68	0.21	15.7	71.7	165.2	2.26
SO2	45.3	29.2	25.5	48.0	99	8.06	4.21	6.32	0.21	17.5	83.6	168.5	1.95
SO3	44.9	30.6	24.5	48.1	99	7.98	4.48	6.03	0.22	15.9	141.4	195.9	1.96
SO4	49.3	28.0	22.7	48.5	99	7.90	4.36	5.95	0.22	15.7	149.6	210.9	2.28
VA1	32.9	40.1	27.0	19.1	54	5.47	2.42	4.53	0.12	21.9	65.0	198.4	2.38
VA2	32.1	42.1	25.8	19.6	40	5.11	2.48	4.70	0.12	22.8	27.3	72.2	2.52
VA3	39.6	28.7	31.7	19.3	66	5.50	2.48	4.54	0.12	22.0	14.5	86.3	2.41
VA4	32.9	34.2	32.9	20.8	89	7.09	2.33	3.86	0.12	18.7	34.0	130.3	2.62
VA5	23.9	39.3	36.8	24.2	58	5.54	2.98	5.64	0.15	21.9	11.0	113.7	3.43
VA6	24.1	45.1	30.8	20.4	74	6.27	2.89	5.47	0.14	22.7	33.3	160.2	3.10
VA7	29.0	48.4	22.6	18.3	64	5.84	2.77	4.97	0.14	20.6	39.5	266.5	2.48
VA8	23.7	44.3	32.0	21.3	35	4.59	2.68	5.08	0.13	22.7	22.0	125.4	2.69

Table S3. PERMANOVA performed on 16S rRNA (A) and ITS (B) Illumina MiSeq datasets, based on Bray-Curtis distances, with 10^4 permutations. Results are presented for all soils and conditions together, and for each soil separately.

A. All treatments				**B.** All treatments			
Source of variation	R^2	F	*P* value	Source of variation	R^2	F	*P* value
Soil	0.42	14.87	< 0.001	Soil	0.6	24.91	< 0.001
Inoculation	0.03	2.26	0.048	Inoculation	0.04	3.3	0.023
Soil × Inoculation	0.05	1.53	0.11	Soil × Inoculation	0.03	1.1	0.316
MI4				MI4			
Source of variation	R^2	F	*P* value	Source of variation	R^2	F	*P* value
Inoculation	0.14	1.53	0.004	Inoculation	0.14	0.63	0.065
MI5				MI5			
Source of variation	R^2	F	*P* value	Source of variation	R^2	F	*P* value
Inoculation	0.21	2.23	0.004	Inoculation	0.17	1.9	0.048
MI2				MI2			
Source of variation	R^2	F	*P* value	Source of variation	R^2	F	*P* value
Inoculation	0.15	1.67	0.009	Inoculation	0.18	2	0.004

Table S4. Relative abundance of the *Fusarium* genus among fungi and of individual *Fusarium* species among the *Fusarium* genus. Asterisks indicate significant difference in the relative abundance of individual *Fusarium* species in inoculated vs. non-inoculated soil based on Kruskal-Wallis tests followed by Fisher's Least Significant Difference (LSD) tests with Bonferroni correction ($P < 0.05$).

Soil	*F. burgessii*	*F. croci*	*F. equiseti*	*F. graminearum*	*F. hostae*	*F. incarnatum*	*F. neocosmosporiellum*	*F. nygamai*	*F. oxysporum*	*F. tricinctum*	*Fusarium* unclassified	% *Fusarium* among total fungal reads
MI4_C	0.00	0.00	2.28	2.63	6.29	0.11	3.75	0.57	76.98	0.71	6.68	9.41
MI4_Fg1	0.03	0.00	1.45*	25.48*	5.40	1.19	2.58	0.54	57.09	1.16	5.08	11.82
MI5_C	0.04	0.00	0.86	0.94	10.23	0.00	2.99	0.31	78.02	2.35	4.27	5.93
MI5_Fg1	0.00	0.00	0.60	29.36*	5.54	0.01	2.09	0.00	58.81	0.24	3.37	5.71
MI2_C	0.00	0.00	19.26	0.53	0.26	0.00	1.87	0.23	66.96	3.31	7.58	6.59
MI2_Fg1	0.00	0.03	17.68	19.98*	0.07	0.00	1.78	0.14	54.07	1.81	4.45*	8.83

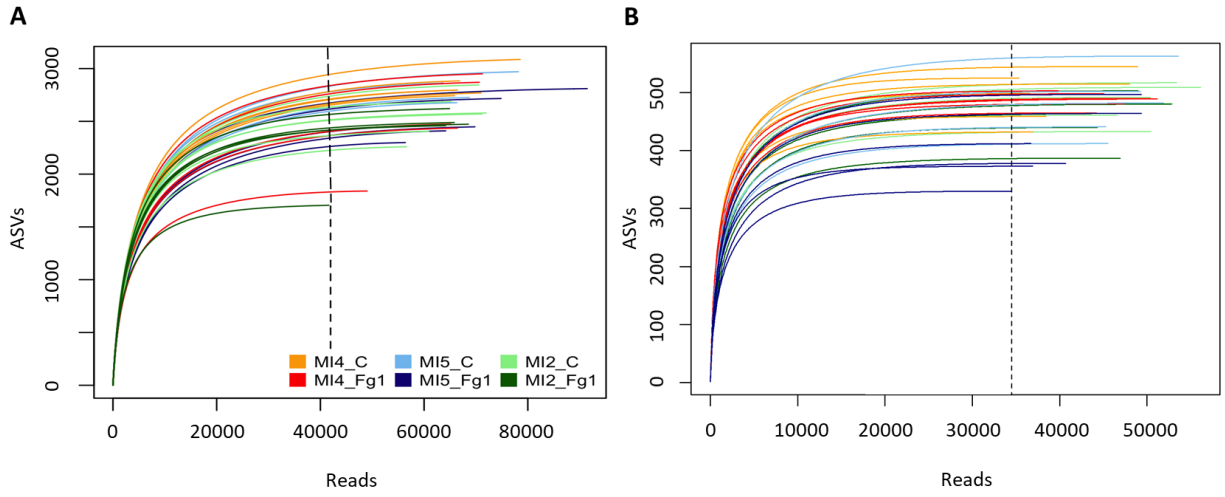

Figure S1. Rarefaction curves with the estimated species richness of each replicate of MI4, MI5 and MI2 rhizospheres for (A) 16S rRNA gene and (B) ITS. Dotted lines represent the rarefaction limit.

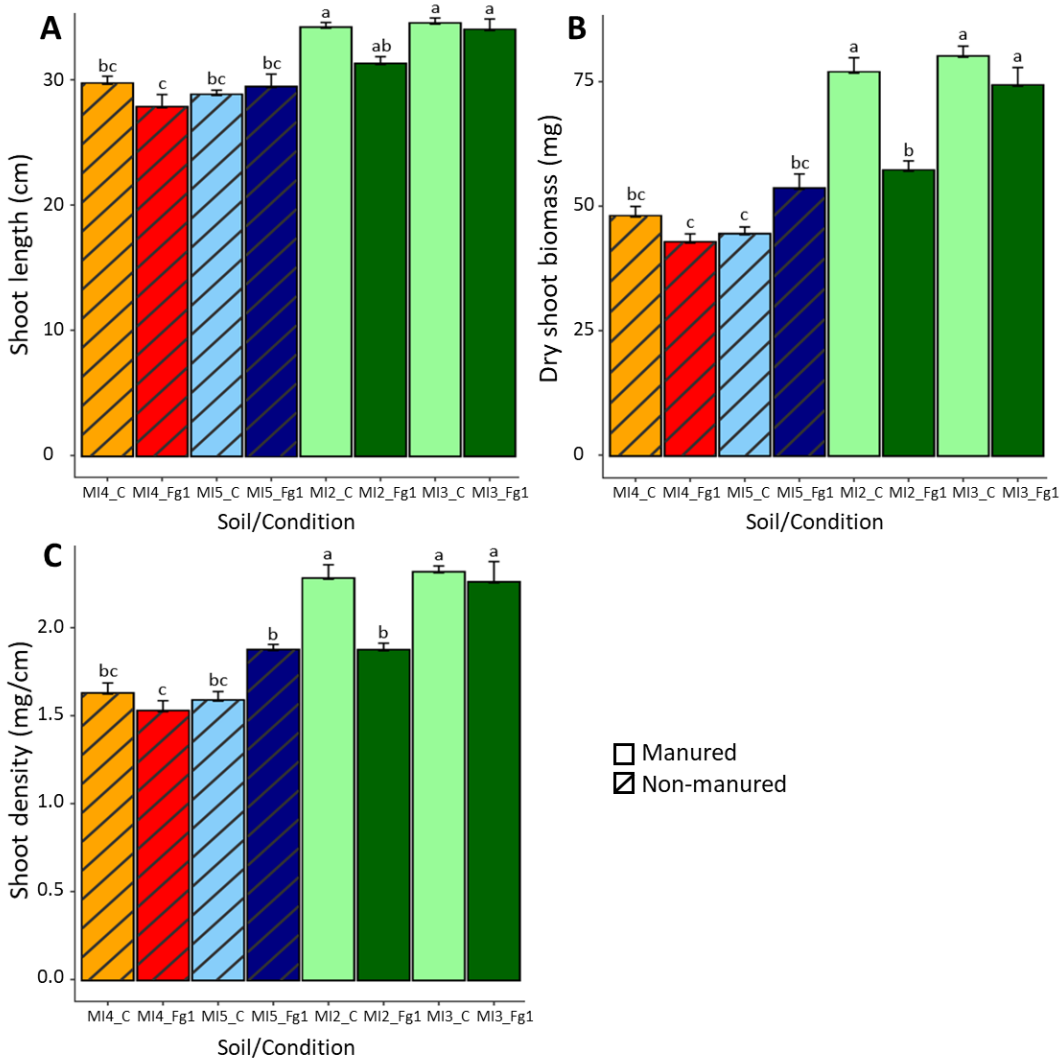

Figure S2. Wheat suppressiveness assay with soils from Mionica (MI4, MI5, MI2 and MI3), non-inoculated (shown as MIi_C) or inoculated with *Fusarium graminearum* Fg1 (shown as MIi_Fg1). Soils that did not receive manure amendments are represented with stripes. All results were obtained at 4 weeks and are presented as means and standard errors (n = 10). Non-germinated plants were regarded as missing data (NA). Data were treated with ANOVA and Tukey's HSD test ($P < 0.05$). Statistical differences are shown with letters a to c. (A) Shoot length (cm). (B) Dry shoot biomass (mg). One plant was discarded from MI2 inoculated soil and one from MI3 inoculated soil because of extreme values. (C) Shoot density (mg/cm). Two plants were discarded from MI2 inoculated soil and one from MI3 inoculated soil because of extreme values.

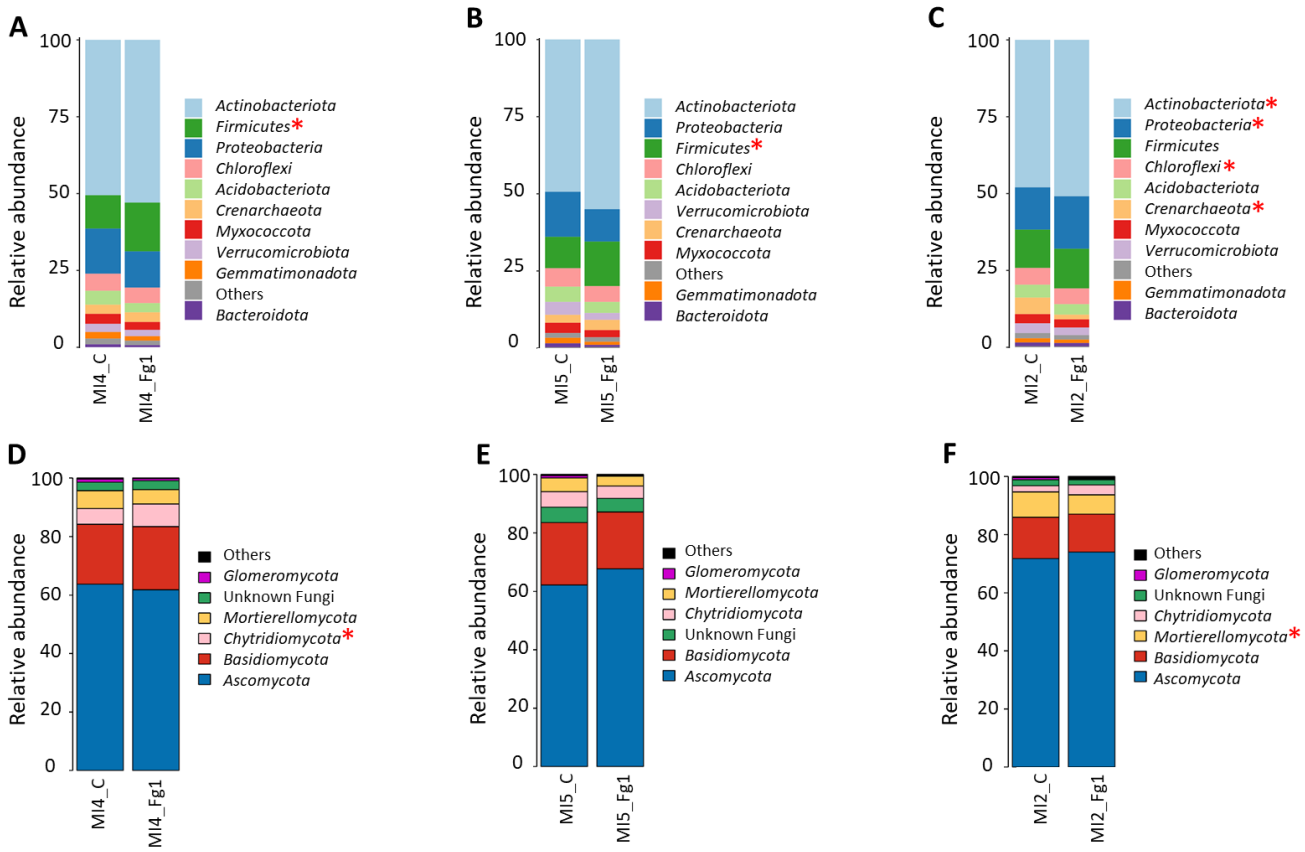

Figure S3. Relative abundance of prokaryotic (A, B, C) and fungal phyla (D, E, F) in the rhizosphere of soils from Mionica MI4, MI5 and MI2. MIi_C, control (non-inoculated soils); MIi_Fg1, *Fusarium graminearum* Fg1-inoculated soils. Asterisks indicate significant differences between each inoculated vs. non-inoculated soil based on Kruskal-Wallis tests followed by Fisher's Least Significant Difference (LSD) tests with Bonferroni correction ($P < 0.05$).

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CHAPTER 3

Genomics of biocontrol bacteria from soils of contrasting suppressiveness status against *Fusarium graminearum*

AVANT-PROPOS

The soil environment serves as a rich reservoir of phytobeneficial bacteria, which have the ability to interact with plants and exert positive effects on their health and development. These bacteria can colonize various niches within the soil, including the rhizosphere, root surfaces, and the surrounding soil matrix (Sánchez-Cañizares et al., 2017). Through intricate interactions with plants, they can enhance nutrient acquisition, stimulate plant growth, and provide protection against phytopathogens. In addition to various fungal strains (Silva et al., 2018), such as those within the *Trichoderma* (Guzmán-Guzmán et al., 2019; Kappel et al., 2020) or *Fusarium* (Park et al., 1988; Forsyth et al., 2006) genera, *Plant Growth-Promoting Rhizobacteria* (PGPR) play a significant role in suppression of diseases caused by phytopathogens. These bacteria employ diverse mechanisms, including antagonism through the production of antimicrobial compounds like hydrogen cyanide, 2,4-diacetylphloroglucinol, and lytic enzymes, as well as competition for resources and parasitism (Nguvo & Gao, 2019; Chapter 1). Furthermore, these beneficial bacteria have a positive impact on plant growth, enhancing the plant fitness and increasing resistance towards abiotic stressors (Oleńska et al., 2020). Additionally, these bacteria induce systemic resistance in plants, thus providing an additional layer of protection against phytopathogens (Lv et al., 2023). Beneficial soil bacteria also contribute to the formation of disease-suppressive soils, with adjacent fields often exhibiting variations in disease suppression levels despite similar climate and agricultural practices (Almario et al., 2014; Chapter 2). These differences can be attributed to variations in the indigenous microbiome present in these soils (Siegel-Hertz et al., 2018; Ossowicki et al., 2020). To the best of our knowledge, PGPR with phytoprotective abilities have typically been isolated from disease-suppressive soils, or at least from soils where no diseases were detected at the time of isolation (Luo et al., 2018; Wang et al., 2021; Fatima et al., 2022). Nowadays, the study of disease suppressiveness in soils often involves metagenomic approaches, as discussed in my previous chapter, as well as culturomic approaches. Culturomics is a high-throughput screening approach for isolates obtained from the environment, which involves isolation of microorganisms on various culture media and their rapid characterization afterwards (Lagier et al., 2016; Sahu et al., 2021). This technique allows for the comparison of cultivable fractions between two conditions, such as dysbiosis in the human gut microbiome, or it could be used to compare microorganisms colonizing suppressive soils with those found in conducive soils (Lagier et al., 2016; Oni et al., 2020). However, much less attention was put on pathogen-suppressive soils, where pathogen growth and development is disrupted. In this chapter, our main focus is to determine whether both pathogen-suppressive (i.e., fungistatic) and pathogen-conducive (i.e., non-fungistatic) soils harbor microorganisms with biocontrol potential.

In the present chapter, our objective was to compare functional, genomic and phytoprotective potential of bacterial isolates originating from fungistatic and non-fungistatic soils, using the MI (MI2, MI3, MI4, MI5; near Mionica; some of them also disease-suppressive) and CA soils (CA1, CA2, CA3; near Čačak; Chapter 2). In order to achieve this, wheat plants were grown in MI and CA soils for 28 days, they were harvested and their rhizospheres were used to isolate representatives of various bacterial genera on both general and selective plating media. 244 isolates were randomly picked, purified and subjected to an *in vitro* confrontation assay with *Fusarium graminearum,* a first screening procedure that enabled us a choice of bacteria for the genome sequencing. After performing the Illumina NovaSeq sequencing, genomes of the chosen bacteria were annotated, searching for genes known to be involved in biocontrol and plant growth promotion and carbohydrate-active enzymes (CAZymes) were predicted using dbCAN2 server. These chosen bacteria were also characterized functionally, by performing *in vitro* assays, including production of HCN and lytic enzymes, and production of ACC deaminase,

phytohormones, siderophores and solubilization of phosphates. They were also assessed for their ability to inhibit *F. graminearum* conidia germination, as well as for their ability to produce VOCs that inhibit *F. graminearum* mycelial growth. Based on the results of *in vitro* confrontation assay with *F. graminearum* and the two latter assays, a subset of isolates was tested in greenhouse conditions for their ability to protect wheat from crown-rot disease caused by *F. graminearum*, using 10 pots x 3 wheat seeds inoculated with *F. graminearum* spore suspension and bacterial suspension for each treatment. Finally, putative biosynthetic gene clusters found in the genomes of these bacteria (tested in a greenhouse experiment) were identified using the antiSMASH and manually curated.

All of this work has led to the drafting of publication "Genomics of biocontrol bacteria from soils of contrasting suppressiveness status against *Fusarium graminearum*" (submission scheduled for the end of 2023).

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GENOMICS OF BIOCONTROL BACTERIA FROM SOILS OF CONTRASTING SUPPRESSIVENESS STATUS AGAINST *FUSARIUM GRAMINEARUM*

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ABSTRACT

The soil-borne fungal pathogen *Fusarium graminearum* is causing significant yield and economic losses, particularly in cereal-growing regions worldwide. However, suppressive soils, where diseases caused by a phytopathogen are controlled, can serve as a source of bacteria with potential biocontrol activity against pathogens. In this study, our aim was to test the hypothesis that fungistatic soils, where pathogen survival and development are disrupted, are valuable for identifying bacteria with biocontrol activity. We used soils fungistatic and non-fungistatic to *F. graminearum*, from two locations in Serbia, namely Mionica and Čačak. From the rhizospheres of wheat plants grown in these soils, we isolated bacteria representing diverse taxonomic groups. We sequenced their genomes and performed *in silico*, *in vitro*, and *in planta* characterizations to estimate their phytoprotective capacity against *F. graminearum*. Our findings suggested that both fungistatic and non-fungistatic soils may serve as sources of biocontrol bacteria, and their whole-genome sequencing information provided insights into the potential mechanisms contributing to their antagonistic properties.

Keywords: fungistasis, PGPR, phytopathogens, rhizosphere, comparative genomics

INTRODUCTION

Soil-borne fungal pathogens cause important crop losses and are difficult to control (Raaijmakers et al., 2009; Katan, 2017; Panth et al., 2020). In this context, the existence of soils that are suppressive to root diseases is of prime interest, because microbial interactions in these soils are effective at protecting crops from phytopathogens infecting roots (Kyselková & Moënne-Loccoz, 2012; Schlatter et al., 2017). This phenomenon of disease suppressiveness has been described for many soil-borne fungal pathogens, including the take-all fungus *Gaeumannomyces graminis* var. *tritici* (Shipton, 1973), *Thielaviopsis basicola* (Stutz et al., 1986), *Rhizoctonia solani* (Mendes et al., 2011) and *Fusarium oxysporum* (Alabouvette, 1986).

Suppressive soils represent a reservoir of microorganisms with phytoprotective potential, and indeed many prominent biocontrol strains originate from suppressive soils, such as *Pseudomonas* sp. Q2-87 (*P. corrugata* subgroup) (Weller et al., 2007), *P. synxantha* 2-79 (formerly *fluorescens*) (Weller & Cook, 1983) and *P. brassicacearum* Q8r1-96 (formerly *fluorescens*) (Raaijmakers & Weller, 1998) isolated from wheat in take-all decline soils, *P. protegens* CHA0 (formerly *fluorescens*) (Stutz et al., 1986) isolated from tobacco in soil suppressive to black root rot disease (causal agent *T. basicola*), and *Pseudomonas* sp. C7 (*P. corrugata* subgroup) (Lemanceau & Alabouvette, 1991) and non-pathogenic *F. oxysporum* Fo47 (Fuchs et al., 1997; Duijff et al., 1998; Fuchs et al., 1999) isolated from soil suppressive to Fusarium wilt of tomato. However, effective biocontrol agents may also originate from conducive soils, as found with *Pseudomonas* bacteria producing the antifungal metabolites hydrogen cyanide (HCN) and 2,4-diacetylphloroglucinol (DAPG) that occur in soils conducive to black root rot (Ramette et al., 2006; Frapolli et al., 2010). Therefore, whether suppressive soils represent a better source of phytoprotective microorganisms is intuitively appealing but remains to be substantiated by considering a wider taxonomic range of candidate biocontrol strains.

Suppressiveness encompasses a range of field conditions, starting with pathogen suppressiveness i.e., the inability of the fungal pathogen to survive and proliferate in soil. Such fungistasis often entails competition with the rest of the soil microbiota (general suppression), along with microbial release of inhibitors (Garbeva et al., 2011; de Boer et al., 2019). Part of the soil microbiota colonizes plant roots and its rhizosphere interactions may confer specific suppressiveness to particular diseases (Weller et al., 2007; Kyselková & Moënne-Loccoz, 2012; Almario et al., 2013; Schlatter et al., 2017). The search for biocontrol agents usually focuses on root-colonizing microorganisms from soils displaying specific suppressiveness, and the usefulness of fungistatic soils has received much less attention, despite the importance of microbial control in these soils. This led us to hypothesize that fungistatic soils could represent a prime source of microorganisms with biocontrol potential.

The objective of this work was to assess the usefulness of fungistatic soils as a source of biocontrol agents against soil-borne pathogens. As models, we chose soils fungistatic to *Fusarium graminearum*, because (i) soils with and without fungistasis towards this pathogen occur (Legrand et al., 2019), (ii) some of them are also suppressive (or not) to Fusarium crown and root-rot disease (Todorović et al., submitted; Chapter 2), (iii) bacteria of contrasted taxonomy may control *F. graminearum*, such as *Pseudomonas* (Hu et al., 2014; Huang et al., 2018), *Bacillus* (Zhao et al., 2014; Zalila-Kolsi et al., 2016), *Paenibacillus* (Zalila-Kolsi et al., 2016) and *Streptomyces* (Bubici, 2018; Colombo et al., 2019), and (iv) *F. graminearum* is an emerging pathogen of high concern in wheat farming (Ma et al., 2013).

To test our hypothesis, we used *F. graminearum*-fungistatic and non-fungistatic soils from two locations in Serbia (Mionica and Čačak), their disease-suppressive status being also

documented in the case of soils from Mionica (Todorović et al., submitted; Chapter 2). Bacteria were isolated from the rhizospheres of wheat grown in these soils, on various general and selective media, and they were tested for the ability to inhibit *F. graminearum in vitro*. The most promising isolates were selected for genome sequencing, the search of genes involved in plant-growth promotion or biocontrol properties, functional determination of plant-beneficial traits, and biocontrol assays *in planta*.

MATERIAL AND METHODS

Isolation of rhizosphere bacteria

Soils from locations near Mionica (MI), Serbia, i.e., MI2, MI3, MI4 and MI5, and from Čačak (CA), Serbia, i.e., CA1, CA2 and CA3 (Table 1), were used for bacterial isolation. During the sampling, the first few cm of topsoil were removed and samples were taken from 5-20 cm depth. Seeds of the winter wheat (*Triticum aestivum* L.) variety Récital were grown in these soils for 28 days, harvested, their roots separated, vigorously shaken and then the rhizosphere sampled using a protocol adapted from Bulgarelli et al. (2012). Briefly, for each soil, wheat root systems with adhering soil were collected in 50 mL of phosphate buffered saline (NaCl, 8 g; KCl, 0.2 g; KH_2PO_4, 0.24 g; Na_2HPO_4, 1.42 g; H_2O, 1000 mL) and shaken for 1 h at 160 rpm (Innova 42R, New Brunswick Scientific, Edison, NJ, USA). The roots were discarded and the suspension centrifuged at 4000*xg* for 20 min. The resulting pellet was suspended in 20 mL of 0.8% NaCl, representing the rhizosphere soil extract.

Table 1. Serbian soils MI and CA used to isolate bacteria, with their fungistasis and suppressiveness status. Some of the soils received manure amendments, whereas the others did not.

Location	Field	GPS coordinates of the fields		Manure amendments	Fungistasis status	Suppressiveness status
Mionica (MI)	MI2	44.24611 N	20.10431 E	Yes	Fungistatic	Suppressive
	MI3	44.24540 N	20.10350 E	Yes	Fungistatic	Suppressive
	MI4	44.24745 N	20.10012 E	No	Non-fungistatic	Conducive
	MI5	44.24759 N	20.09931 E	No	Non-fungistatic	Suppressive
Čačak (CA)	CA1	43.89897 N	20.54435 E	No	Non-fungistatic	Not determined
	CA2	43.89910 N	20.54450 E	Yes	Non-fungistatic	Not determined
	CA3	43.89905 N	20.54312 E	No	Fungistatic	Not determined

Bacteria were isolated by plating serially diluted rhizosphere extracts on general media, i.e., nutrient agar (NA; Carl Roth, Karlsruhe, Germany) and Tryptone Soya Agar (TSA; Carl Roth), as well as on selective media: Citrimide Agar (Merck, Darmstadt, Germany) and King's B agar (Condalab, Madrid, Spain) for *Pseudomonas*, Fiodorov agar (Anderson, 1958) for *Azotobacter*, and

Starch Ammonia Agar (SAA; starch, 10 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4$ x $7H_2O$, 1 g; NaCl, 1 g; KNO_3, 1 g; $CaCO_3$, 3 g; agar, 20 g; H_2O, 1000 mL) for *Actinomycetes*. Part of the rhizosphere extract was pasteurized at 80°C for 10 min and plated on NA to isolate members of the genus *Bacillus* (NAsp). All plates were incubated at 28°C in the dark until bacterial growth occurred, individual colonies were picked and purified until pure cultures were obtained.

Fusarium graminearum fungal strain and preparation of spore suspension

The highly virulent and toxin-producing *Fusarium graminearum* MDC_Fg1 isolate (throughout the text as *F. graminearum* Fg1) used in the experiments was isolated from naturally infected cereal grains in northern France (Alouane et al., 2018). *F. graminearum* Fg1 spore suspension was prepared by growing the fungus in Mung Bean Broth (MBB) (Evans et al., 2000) for 6 days at 22°C with shaking at 180 rpm (Incubator Shaker Series I26, New Brunswick Scientific Co., Inc., Edison, New Jersey, USA). After incubation, a volume of the preculture was taken and diluted to one tenth in fresh MBB medium and incubated for 10 days under the same conditions. The resulting culture was vortexed, filtered to discard mycelium and centrifuged at 4700*xg* for 10 min (Avanti J-E Series, Beckman Coulter, Fullerton, USA). Supernatant was discarded and the resulting pellet was washed twice with sterile water. Titration of spores in the suspension was estimated using a Thoma counting chamber.

Confrontation assay of rhizosphere isolates and *Fusarium graminearum*

Confrontation assay with isolated rhizosphere bacteria and *F. graminearum* Fg1 was carried out on PDA plates (Potato Dextrose Agar; Condalab). PDA plates were inoculated with discs (Ø7 mm) taken from the edges of an 8-day-old *F. graminearum* Fg1 colony and a streak of each bacterium from the bacterial collection was made 3 cm apart from the pathogen. In the case of *Actinomycetes*, firstly bacteria were inoculated on PDA plates, and after 5 days, fungal discs were added 3 cm apart from the bacteria. Control plates were inoculated with *F. graminearum* Fg1 discs only. Plates were incubated in the dark at 22°C for 7 days, when observed changes in pathogen growth were noted, and for 14 days, when changes in colony morphology, were noted. The percentage of inhibition of pathogen growth was calculated according to a formula by Siripornvisal (2010), i.e., $I\% = (r_o\text{-}r)/r_o \times 100$, where I% is percentage of growth inhibition; r_o is the radius of the *F. graminearum* Fg1 colony on a control dish and r is the radius of *F. graminearum* Fg1 inhibited by the bacteria. Bacteria able to inhibit mycelial growth by more than 50% or alter fungal colony morphology were considered as antagonistic.

DNA extraction, genome sequencing and assembling

Genomic DNA was extracted from an overnight culture, using a Nucleospin tissue kit (Macherey-Nagel, France), according to the manufacturer's instructions. For the whole genome sequencing, genomic DNA library preparation and sequencing was performed at Novogene (Cambridge, England), using Illumina NovaSeq 6000 technology. Genomic DNA was randomly sheared into short fragments. The resulting fragments were end repaired, A-tailed and further ligated with Illumina adapters to generate a 2x150-bp paired-end library. The adapter-ligated fragments were PCR amplified, size selected, purified and sequenced. The original raw data from the Illumina platform were converted to sequenced reads, by base calling. The raw data were recorded in a FASTQ file, which contains the sequencing reads and the corresponding sequencing quality. Fastp

software (v.0.23.1; Chen et al., 2018) was used for trimming sequences (default settings) and Unicycler software (v.0.5.0; Wick et al., 2017) (default settings) was used for *de novo* assembly. Identification and construction of phylogenetic trees was performed with the Type strain Genome Server (TYGS) (https://tygs.dsmz.de/; Meier-Kolthoff & Göker, 2019; Meier-Kolthoff et al., 2022). Genomic features of the isolates were obtained using the MicroScope platform (Vallenet et al., 2020).

Genome annotation

Genome annotation was done automatically with the MicroScope platform (v.3.15.4; Vallenet et al., 2020). Diamond blastp (v.2.0.8.146; Buchfink et al., 2015), was used to search for genes known to be involved in biocontrol and plant-growth promotion (accession numbers available in Table S1) within genome protein sequences using the options --query-cover 80 --id 70, in order to filter the hits with minimum 80% query coverage and minimum 70% amino acid identity.

The searched functions included (i) production of antimicrobial compounds phenazine (*phzABCDEFG*) (Dar et al., 2020), 2-hexyl-5-propyl-alkylresorcinol (*darABC*) (HPR; Nowak-Thompson et al., 2003), DAPG (*phlABCD*) (Bangera & Thomashow, 1999), pyrrolnitrin (*prnABCD*) (Kirner et al., 1998), HCN (*hcnABC*) (Ramette et al., 2003) and pyoluteorin (*pltABCDEFGLM*) (Nowak-Thompson et al., 1999), (ii) production of insect toxin FitD (*fitD*) (Loper et al., 2012) and alkaline metalloproteinase AprA (*aprA*) (Loper et al., 2012) involved in biocontrol, (iii) production of siderophores pyoverdine (*pvdL*) (Schalk & Guillon, 2013), pyochelin (*pchABCDEF*) (Reimmann et al., 2001) and pseudomonine (*pmsABCE*) (Matthijs et al., 2009), (iv) signaling and modulation of plant hormonal balance by deamination of ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (*acdS*) (Shah et al., 1998), ethylene production (*efe*) (Wang et al., 2010), auxin biosynthesis (*iaaMH*, *ipdC*/*ppdC*) (Loper et al., 2012; Gruet et al., 2022), auxin catabolism (*iacABCDEFGHI*) (Loper et al., 2012), acetoin synthesis (*budB*/*ilvNB*/*alsS*, *budA*/*alsD*) (Blomqvist et al., 1993; Loper et al., 2012), 2,3-butanediol synthesis (*budC*/*ydjL* in addition to the acetoin synthesis genes) (Nicholson, 2008), 2,3-butanediol conversion to acetoin (*adh*/*bdhA*/*ydjL*) (Huang et al., 1994; Nicholson, 2008), acetoin catabolism (*acoABCX*) (Huang et al., 1994), (v) transformation of P and N sources by phosphate solubilization (*gcd*, *gad*) (Miller et al., 2010), nitrogen fixation (*nifHDK*) (Bruto et al., 2014) and denitrification (*nirK*, *nirS*) (Bruto et al., 2014; Coyne et al., 1989). In case presence of more than one gene is necessary to achieve a function (e.g., presence of both *iaaM* and *iaaH* for the synthesis of auxin via indole-3-acetamide pathway) and only some of the necessary genes were found in the genome, we checked for the presence of the missing genes with less stringent blast result filtering criteria (--query-cover 80 --id 30). Putative biosynthetic gene clusters were further identified using the antiSMASH (Blin et al., 2019) within the MicroScope platform and the annotations were manually curated.

Carbohydrate-active enzymes (CAZymes) were predicted using dbCAN2 (v.3; Zhang et al., 2018) and compared with the CAZy database using HMMER (v.3.3; Eddy, 2011). Prediction of function and substrate specificity of CAZyme families or subfamilies was performed based on a review of activities assigned to CAZymes with known structures (characterized enzymes) in the CAZy database (http://www.cazy.org) (Lombard et al., 2014) and manually curated, as previously described (López-Mondéjar et al., 2022). Heatmap based on CAZyme counts was generated by pheatmap 1.0.12 R package (Kolde, 2019).

Screening of rhizosphere isolates for their plant-growth promoting and biocontrol properties

Screening of isolates for their plant-growth promoting and biocontrol properties included production of HCN (Bakker & Schippers, 1987) and production of lytic enzymes i.e., extracellular protease on milk agar, production of chitinase tested on Minimal media (Kim et al., 2003), supplemented with 10 % colloidal chitin solution, prepared as described by Murthy & Bleakley (2012), and production of cellulase on media containing Carboxymethyl cellulose (CMC; Sigma Aldrich, USA) (Teather & Wood, 1982; Chantarasiri, 2014).

The ability of isolates to solubilize inorganic P sources was tested on NBRIP media (National Botanical Research Institute's Phosphate; Nautiyal, 1999), and their ability to produce siderophores was tested according to Pérez-Miranda et al. (2007).

Screening also included testing of metabolites involved in modulation of plant hormonal balance, such as ACC deaminase production, tested according to a protocol by Penrose & Glick (2003), which detects α-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. Screening for production of (i) seven auxin phytohormones, i.e., indole-3-acetic acid (IAA), indole-3-lactic acid, indole-3-carboxylic acid, indole-3-pyruvic acid, indol-3-butyric acid (IBA), tryptophol and indole-3-propionic acid, (ii) five cytokinins, i.e., trans-zeatin, trans-zeatin riboside (ZR), kinetin, 6-benzylaminopurine (BAP) and isopentenyl adenosine (IPA), (iii) two gibberellins, i.e., gibberellin A1 (GA1) and gibberellic acid (GA3), (iv) abscisic acid (ABA) and (v) kynurenic acid was done by Ultra High Performance Liquid Chromatography (UHPLC). Briefly, all isolates were grown for 3 days at 28°C (300 rpm) in 2 mL of M9 minimal medium (Miller, 1972) supplemented with 0.4 mM of tryptophan and 0.1 mM of adenine. The cultures were centrifuged at 4500*xg* during 8 min and filtered at 0.2 µm. Supernatants were lyophilized (Alpha 1–4 LSC Martin Christ, Osterode, Germany) for 24 h, the powder obtained was extracted two times with methanol, drying with speed-vac (Centrivap Cold Trap Concentrator LABCONCO, Kansas City, MO, USA), and UHPLC separation was performed with an Agilent 1290 Series instrument (Agilent Technologies France, Les Ulis, France) using a 100 × 3 mm reverse phase column (Agilent Poroshell 120 EC-C18, 2.7 µm particle size). Samples (3 µL) were loaded onto the column equilibrated with solvent A (water + 0.4% formic acid) and solvent B (acetonitrile) in a 98:2 ratio. Compounds were eluted by increasing the acetonitrile concentration to 40% over a 6 min period, then to 100% over 4 min, followed by an isocratic step of 2 min, at a flow rate of 0.5 $mL.min^{-1}$. Hormones were detected with a diode array detector (DAD) and an Agilent 6530 Q-TOF mass spectrometer in positive and negative electrospray ionization, based on comparison with commercial standards on both mass and UV (between 190 and 600 nm) chromatograms, along with accurate mass and UV spectra.

The effect of volatile organic compounds on *Fusarium graminearum* growth

Inhibitory effect of Volatile Organic Compounds (VOCs) produced by rhizosphere isolates towards *F. graminearum* Fg1 was assessed in a system of two Petri dishes sealed together with parafilm. For this assay, 30 µL of each bacterial suspension of optical density 1 (OD 600nm) (Ultrospec 10 Cell Density Meter; Amersham Biosciences, Little Chalfont, UK) was spread onto TSA plate. PDA plates were center-inoculated with discs (Ø7 mm) taken from the edges of 8-days old *F. graminearum* Fg1 colony. After 24 h of bacterial and fungal growth, at 28°C and 22°C, respectively, the lid of TSA plate with bacteria was replaced with a plate containing *F. graminearum* Fg1 and the two plates were firmly sealed together with parafilm. Control plates

were prepared in the same way, but without the bacteria in the bottom plate. Such sealed plates were incubated at 22°C, and the observations were recorded after 72 h. The mycelial growth inhibition (%) of the fungus was determined according to Trivedi et al. (2008), using the formula $(1-r_2/r_1) \times 100$, where r_1 represents the radial growth of *F. graminearum* Fg1 in control plates, and r_2 in plates with bacteria.

Inhibitory effect of bacterial exudates on *Fusarium graminearum* conidia germination

Antagonism potential of bacterial exudates on *F. graminearum* Fg1 spore germination, was tested in a microplate test, according to a protocol by Besset-Manzoni et al. (2019). Supernatant of each tested bacteria was prepared from an overnight TSB (Tryptic Soy Broth; Carl Roth) culture and filtered at 0.2 µm. *F. graminearum* Fg1 spore suspension was prepared by growing the fungus in MBB, as described above. For each assay, 100 µL of the bacterial supernatant, 100 µL of PDB (Potato Dextrose Broth; Condalab), and 50 µL of Fg1 spores at 10^4 spores.mL^{-1} were added in microplate wells, in triplicates. For positive control, 100 µL of TSB was used instead of bacterial supernatants, and for negative control, 50 µL of PDB was used instead of spore suspension. After incubating microplates for 5 days at 28°C, the turbidimetry was measured at 492 nm using an Infinite M200 Pro microplate reader (TECAN, Mannedorf, Switzerland), the value of negative control was subtracted from each bacterial treatment and compared to the positive control.

***In planta* protection assay**

The *in planta* protection assay was carried out in a plant growth chamber (FitoClima, 10.000 EH, ARALAB, Rio de Mouro, Portugal), under the following conditions: 16h day at 20°C/8 h dark at 18°C and 80 % relative humidity. The isolates were used with a neutral soil (without documented suppressiveness) taken in La Côte-Saint-André, France (soil LCSA; 45.37861 N and 5.26722 E; Bouffaud et al., 2016). For each treatment, 30 seeds of wheat cultivar Sumai 3 were distributed in 10 pots (8 x 6 x 6 cm), each filled with 150 g of sterile soil (autoclaved twice at 121°C for 20 min, 24h interval) LCSA. For each *in vivo* assay, bacteria were prepared from an overnight liquid culture, by resuspending harvested bacterial pellets in 10 mM $MgSO_4$ and adjusting the optical density at 600 nm (OD600nm) to 1 (i.e., 10^8 cells.mL^{-1}) (Ultrospec 10 Cell Density Meter; Amersham Biosciences, Little Chalfont, UK). *F. graminearum* Fg1 spore suspension was prepared by growing the fungus in MBB, and adjusted to 10^6 spores.mL^{-1}. Bacteria were inoculated directly onto each seed (10^7 cells per seed) with 100 µL of prepared suspensions. The same day, 100 µL of *F. graminearum* Fg1 spores (10^5 spores per seed) were added directly onto seeds. As a negative control, 30 uninoculated seeds were used, while 30 seeds inoculated only with *F. graminearum* Fg1 spores served as a positive control. Plants were watered every 3 days by adding the water under the pots (watering by capillary movement of water).

At 14 days, the number of germinated seeds was counted. After 45 days of the plant growth, the plants were harvested and measurements were performed, such as: (i) shoot biomass (mg), (ii) the chlorophyll rate of each wheat plant containing three measurements of the 5th, 6th and 7th grown leaf using the SPAD 502 plus device (Minolta Camera Co., Osaka, Japan) and (iii) the disease symptoms of crown-rot on each wheat collar using a 1 to 7 notation index, as follows: 1 = no symptoms, 2 = several non-connected, dark spots only at the collar base, 3 = several non-connected, dark spots, rising up the collar base, 4 = several connected, dark spots, 5 = several connected, dark spots, rising up to several cm, 6 = collar covered with necrosis, collar base very fragile, and 7 = dead plant.

Statistical analyses

All statistical analyses of the greenhouse experiment were performed at $P < 0.05$, using the R 4.2.1. software (https://www.r-project.org). The greenhouse experiment followed a randomized block design, with 10 replicates (i.e., 10 pots). The data did not display normal distribution and homogeneity of variance, based on Shapiro and Levene tests, respectively. For the number of germinated seeds at 14 days, plant biomass and wheat symptoms at 45 days, Kruskal-Wallis tests and post-hoc Dunn's tests were used to compare treatments. For chlorophyll content, the data displayed normal distribution and homogeneity of variance, so an ANOVA followed by Tukey's test was used. For biomass, symptoms and chlorophyll content, the plants that did not germinate were regarded as missing data (NA). All results were presented as mean + standard error. For each plant growth parameter, letters a-d are used to show the statistical relationship between the treatments.

RESULTS

Antagonistic activity of rhizosphere bacteria against *Fusarium graminearum*

Two hundred and forty-four bacteria were isolated from the rhizospheres of wheat plants grown in MI or CA soils, i.e., 118 from fungistatic soils (soils MI2, MI3 and CA3) and 126 from non-fungistatic soil (soils MI4, MI5, CA1 and CA2) (Table 2). Tests based on their ability to inhibit mycelial growth or alter colony morphology of *F. graminearum* Fg1 *in vitro* resulted in the selection of respectively 12 and 11 antagonistic isolates (none exhibiting both effects), making a total of 23 isolates (9.4%). These 23 antagonistic isolates included 10 isolates from fungistatic soils and 13 from non-fungistatic soils, and 13 of the 23 originated from MI soils (6 from fungistatic soils MI2, MI3 and 4 from non-fungistatic MI5, also suppressive to *F. graminearum* disease, versus 3 from non-fungistatic soil MI4, which is not suppressive) (Table 2). For example, isolate IT-7CA2 from non-fungistatic soil CA2 inhibited the growth of *F. graminearum* Fg1 by 95% (Figure S1).

Table 2. Number of isolates obtained from each fungistatic soil (MI2, MI3 and CA3) and non-fungistatic soil (MI4, MI5, CA1 and CA2), with the corresponding isolation media, and number of antagonistic isolates (i.e., isolates that inhibited mycelial growth or altered colony morphology of *Fusarium graminearum* Fg1). NA: Nutrient agar; NAsp: Nutrient agar plated with pasteurized soil sample, aiming to isolate sporogene bacteria; TSA: Tryptone soya agar; KB: King's B; C: Cetrimide agar; F: Fiodorov agar; SAA: Starch ammonia agar.

Soil	**Total number of isolates**								**Number of antagonistic isolates**							
	NA	NAsp	TSA	KB	C	F	SAA	Total	NA	NAsp	TSA	KB	C	F	SAA	Total
Fungistatic soils																
MI2	12	8	10	8	3	5	6	**52**		1	1					**2**
MI3	11	8	9	3	5	4	2	**42**		1	2		1			**4**
CA3	6	5	2	4	5		2	**24**	1			1	2			**4**

Non-fungistatic soils															
MI4	7	6	8	8	4	5	3	**41**	1				1	1	**3**
MI5	10	7	8	9	2	4	1	**41**	1			1	1	1	**4**
CA1	7	4	6	2	4		5	**28**		1				1	**2**
CA2	2	3	4	2	5			**16**		1			1	2	**4**

In summary, antagonistic bacterial isolates were obtained in similar numbers from fungistatic and non-fungistatic soils (and for MI soils, they were obtained from both suppressive and non-suppressive soils).

Genome sequencing of antagonistic rhizosphere bacteria

All 23 genome-sequenced antagonistic isolates differed from one another. Digital DNA-DNA hybridization values (dDDH; computed with GGDC 3.0 and formula 2) of the 23 strains with their closest described type strains (available at the TYGS database, Meier-Kolthoff & Göker, 2019; Meier-Kolthoff et al., 2022), revealed 8 novel genomospecies (hereafter *GS-1* to *GS-8*; Table 3) based on dDDH values below the 70% threshold for species delineation (Chun et al., 2018).

Table 3. Digital DNA-DNA hybridization (dDDH) values of the 8 sequenced antagonistic strains and their closest described species (available at the TYGS database), whose dDDH values are below the 70%, recommended for bacterial species delineation. dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0; Meier-Kolthoff & Göker, 2019; Meier-Kolthoff et al., 2022), using the recommended BLAST method, and formula 2.

Species name from TYGS	**Field**	*B. pseudomycoides* DSM 12442	*Chryseobacterium aureum* 17S1E7	*Brevibacillus porteri* NRRL B-41110	*Burkholderia pyrrocinia* DSM 10685	*P. farris* SWRI79	*P. jessenii* DSM 17150	*P. koreensis* LMG 21318	*P. germanica* FIT 28
Isolates from fungistatic soils									
Bacillus GS-1 IT-79MI2	MI2	68.4							
Isolates from non-fungistatic soils									
Chryseobacterium GS-2 IT-36CA2	CA2		35.1						
Brevibacillus GS-3 IT-7CA2	CA2			58.7					
Burkholderia GS-4 IT-111MI5	MI5				61.7				
Pseudomonas GS-5 IT-194MI4	MI4					43.2			
Pseudomonas GS-6 IT-196MI5	MI5						48.3		
Pseudomonas GS-7 IT-93MI4	MI4							43.5	
Pseudomonas GS-8 IT-232MI5	MI5								48.0

* Strains that are not presented in the table as their dDDH values were >70%, are the following: *Bacillus licheniformis* IT-74MI3, *Bacillus pseudomycoides* IT-19CA3, *Bacillus pseudomycoides* IT-40CA3, *Priestia megaterium* IT-180MI3, *Priestia megaterium* IT-210MI2, *Kosakonia quasisacchari* IT-91MI3, *Pseudomonas donghuensis* IT-53CA3, *Pseudomonas chlororaphis* IT-51CA3, *Pseudomonas chlororaphis* IT-162MI3, *Bacillus licheniformis* IT-13CA1, *Bacillus velezensis* IT-133MI5, *Burkholderia ambifaria* IT-158MI4, *Pseudomonas soli* IT-47CA2, *Pseudomonas chlororaphis* IT-48CA2 and *Pseudomonas brassicacearum* IT-43CA1.

Whole-genome phylogenetic tree showed that the 23 antagonistic strains were distributed across three phyla and seven genera (Figure 1).

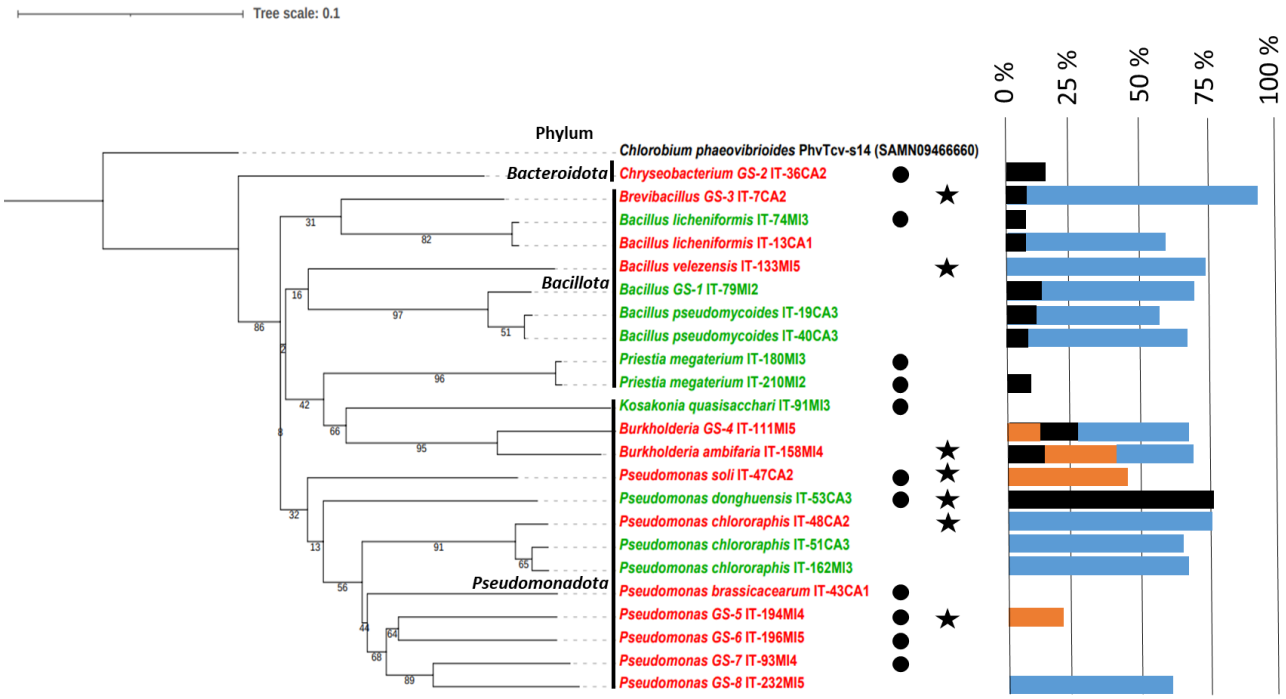


Figure 1. Whole genome-based phylogenetic tree for 23 antagonistic bacteria from fungistatic (green) and non-fungistatic soils (red), and ability to affect *Fusarium graminearum* Fg1 colony morphology (black circle), to inhibit *Fusarium graminearum* Fg1 conidia germination (black bars), to inhibit *Fusarium graminearum* Fg1 by bacterial VOCs (orange bars), and to inhibit *Fusarium graminearum* Fg1 mycelial growth in a dual-culture assay (blue bars). Black stars indicate isolates chosen for *in planta* assay. The tree was inferred with FastME 2.1.6.1 (Lefort et al., 2015) from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences, and visualized using iTOL software (Letunic & Bork, 2021). Branch numbers are GBDP pseudo-bootstrap support values from 100 replications, with an average branch support of 56.9 %. *Chlorobium phaeovibrioides* PhvTcv-s14 (BioSample accession number: SAMN09466660) was used as the outgroup.

The 10 strains from fungistatic soils belonged to the phyla *Pseudomonadota* (formerly *Proteobacteria*) (3 strains from the genus *Pseudomonas* and 1 from the genus *Kosakonia*) or *Bacillota* (formerly *Firmicutes*) (4 strains from the genus *Bacillus* and 2 from the genus *Priestia*) (Figure 1). The 13 strains from non-fungistatic soils belonged to the phyla *Pseudomonadota* (7 from *Pseudomonas* and 2 from *Burkholderia*), *Bacillota* (2 from *Bacillus* and 1 from *Brevibacillus*), as well as *Bacteroidota* (formerly *Bacteroidetes*) (1 from *Chryseobacterium*). Their genomic features are presented in Table 4.

Table 4. Genomic features of the 23 antagonistic bacteria whose genomes were sequenced in this study.

Species name from TYGS	Isolate name	Field of isolation	Genome size (bp)	Plasmid	GC-content (%)	No. contigs	Coding DNA sequences (CDS)
Isolates from fungistatic soils							
			***Bacillota* (formerly *Firmicutes*)**				
Bacillus licheniformis	IT-74MI3	MI3	4,240,635	-	45.92	18	4619
Bacillus GS-1	IT-79MI2	MI2	5,465,265	+	35.44	139	6013
Bacillus pseudomycoides	IT-19CA3	CA3	4,323,109	+	35.75	79	4603
Bacillus pseudomycoides	IT-40CA3	CA3	3,061,249	+	35.83	36	3223
Priestia megaterium	IT-180MI3	MI3	5,635,521	+	37.87	44	6194
Priestia megaterium	IT-210MI2	MI2	5,379,042	+	37.85	28	5699
			***Pseudomonadota* (formerly *Proteobacteria*)**				
Kosakonia quasisacchari	IT-91MI3	MI3	5,073,466	-	53.48	70	4744
Pseudomonas donghuensis	IT-53CA3	CA3	5,663,148	-	62.45	59	5408
Pseudomonas chlororaphis	IT-51CA3	CA3	6,957,669	-	62.92	29	6655
Pseudomonas chlororaphis	IT-162MI3	MI3	6,686,366	-	63.10	21	6340
Isolates from non-fungistatic soils							
			***Bacteroidota* (formerly *Bacteroidetes*)**				
Chryseobacterium GS-2	IT-36CA2	CA2	5,012,043	-	35.62	27	4631
			***Bacillota* (formerly *Firmicutes*)**				
Brevibacillus GS-3	IT-7CA2	CA2	6,478,916	+	47.11	55	6303
Bacillus licheniformis	IT-13CA1	CA1	4,332,481	-	45.75	54	4751
Bacillus velezensis	IT-133MI5	MI5	3,857,335	-	46.57	38	3742
			***Pseudomonadota* (formerly *Proteobacteria*)**				
Burkholderia GS-4	IT-111MI5	MI5	7,802,089	-	66.69	73	7775
Burkholderia ambifaria	IT-158MI4	MI4	7,617,524	-	66.61	75	7516
Pseudomonas soli	IT-47CA2	CA2	5,708,236	-	63.78	72	5500
Pseudomonas chlororaphis	IT-48CA2	CA2	6,818,347	-	62.98	60	6537
Pseudomonas brassicacearum	IT-43CA1	CA1	6,737,027	-	60.86	70	6361
Pseudomonas GS-5	IT-194MI4	MI4	6,582,923	-	59.39	77	6261
Pseudomonas GS-6	IT-196MI5	MI5	6,303,596	-	59.61	84	6018
Pseudomonas GS-7	IT-93MI4	MI4	6,106,124	-	60.32	58	5645
Pseudomonas GS-8	IT-232MI5	MI5	6,512,142	-	59.15	61	6073

In summary, most antagonistic bacterial strains from fungistatic or non-fungistatic soils belonged to the *Pseudomonadota* or *Bacillota* phyla (and for MI soils, *Pseudomonadota* antagonistic strains were obtained from both suppressive and non-suppressive soils).

Presence of genes involved in biocontrol and plant growth promotion

The 13 genome-sequenced antagonistic *Pseudomonadota* included 10 *Pseudomonas* strains. In the latter, genes for the production of HCN, pyoverdine, extracellular alkaline protease, ethylene, auxin, for conversion of 2,3-butanediol to acetoin and further acetoin catabolism, and for phosphate solubilization and denitrification were evidenced in 10 *Pseudomonas* strains, regardless of whether they originated from fungistatic or non-fungistatic soils (Table 5). *P. brassicacearum* IT-43CA1 (from non-fungistatic soil) carried genes for the production of DAPG (the presence of the whole operon was confirmed by antiSMASH) and ACC deaminase, whereas the three *P. chlororaphis* strains (from fungistatic or non-fungistatic soils) displayed genes for the production of phenazine, HPR and pyrrolnitrin. In addition, *P. chlororaphis* IT-48CA2 (from non-fungistatic soil) had the gene encoding the insect-toxin FitD. Annotation of CAZymes showed that all the *Pseudomonas* genomes had genes encoding potential chitinases (except *Pseudomonas GS-6* IT-196MI5), as many as 5 in *P. chlororaphis* IT-51CA3 and IT-162M3 (from fungistatic soils) (Figure S2). *P. donghuensis* IT-53CA3, *P. chlororaphis* IT-51CA3 and IT-162MI3 (from fungistatic soils) and *P. soli* IT-47CA2, *P. chlororaphis* IT-48CA2, *Pseudomonas GS-6* IT-196MI5, *GS-7* IT-93MI4 and *GS-8* IT-232MI5 (from non-fungistatic soils) contained copies of the AA10 family, which includes lytic polysaccharide monooxygenases (LPMOs) that potentially target chitin (Figure S3). Genes coding for beta-glucanases were detected in four strains (from both fungistatic and non-fungistatic soils) and cellulase genes in *Pseudomonas GS-6* IT-196MI5 and *GS-5* IT-194MI4 (from non-fungistatic soils), but mannanase genes were not detected.

Table 5. Distribution of genes involved in biocontrol and plant-growth promotion in the 23 bacterial isolates studied in this paper. Presence of the property (the whole gene cluster) is marked with +, and when for certain property there are several possible pathways to achieve a function, names of the genes found in the genome are indicated. Genes are found with DIAMOND blastp (v.2.0.8.146; Buchfink et al. 2015), using the options --query-cover 80 --id 70 (query coverage >80%; amino acid identity >70%), if not specified otherwise.

Isolate name	Phenazine production	HPR production	2,4-DAPG production	Pyrrolnitrin production	HCN production	Pyoverdine production	Ethylene production	ACC deaminase	Auxin biosynthesis	Acetoin biosynthesis	2,3-butanediol biosynthesis	2,3-butanediol conversion to acetoin	Acetoin catabolism	Phosphate solubilization	Nitrogen fixation	Denitrification	Alkaline metalloproteinase production	Insect-toxin FitD
Isolates from fungistatic soils																		
Bacillus licheniformis																		
IT-74MI3										*alsSD*								
Bacillus GS-1																		
IT-79MI2																		
Bacillus pseudomycoides																		
IT-19CA3																		
IT-40CA3																		
Priestia megaterium (formerly *Bacillus megaterium*)																		
IT-180MI3																		
IT-210MI2																		
Kosakonia quasisacchari																		
IT-91MI3										*budBA*	*budBAC*				*+**			
Pseudomonas donghuensis																		
IT-53CA3					*+**				*ipdC*			*adh*	+	*gcd*		*nirS*		
Pseudomonas chlororaphis																		
IT-51CA3	+	+		+	+	+	+		*iaaMH*			*bdhA, adh*	+	*gcd, gad*		*nirK*	+	
IT-162MI3	+	+		+	+	+	+		*iaaMH*			*bdhA, adh*	+	*gcd, gad*		*nirK*	+	
Isolates from non-fungistatic soils																		
Chryseobacterium GS-2																		
IT-36CA2																		

Brevibacillus GS-3																						
IT-7CA2														ydjL								
Bacillus licheniformis																						
IT-13CA1										*alsSD*												
Bacillus velezensis																						
IT-133MI5										*alsSD*	*alsSD, ydjL*			*ydjL*								
Burkholderia GS-4																						
IT-111MI5			+				+							*adh*	+*	*gad*						
Burkholderia ambifaria																						
IT-158MI4			+				+							*adh*	+*	*gad*						
Pseudomonas soli																						
IT-47CA2				+*					*ipdC*							*gad*			*nirK*			
Pseudomonas chlororaphis																						
IT-48CA2	+	+		+	+	+	+		*iaaMH*					*bdhA*		*gcd, gad*			*nirK*	+	+	
Pseudomonas brassicacearum																						
IT-43CA1		+		+			+		*iaaMH**					*adh*	+				*nirS*	+		
Pseudomonas																						
GS-5 IT-194MI4				+	+	+								*adh*	+	*gcd, gad*			*nirS*	+		
GS-6 IT-196MI5						+								*adh*		*gcd*						
GS-7 IT-93MI4				+	+	+										*gcd, gad*				+		
GS-8 IT-232MI5				+	+	+										*gcd, gad*				+		

Genes (and functions) that were searched for in the 23 bacterial isolates, but were not found: *pltABCDEFGLM* (production of pyoluteorin), *pchABCDEF* (production of pyochelin), *pmsABCE* (production of pseudomonine) and *iacABCDEFGHI* (auxin catabolism).

**hcnA* found with <70 % identity (63 % for isolate IT-47CA2 and 69 % for isolate IT-53CA3); *iaaH* found with only 33 % identity for isolate IT-43CA1; *acoX* and *acoR* found with 49 % and 57 % identity, respectively, for isolates IT-158MI4 and IT-111MI5; *nifD* and *nifK* found with 66 % and 49 % identity, respectively, in isolate IT-91MI3, but whole *nif* operon found in the genome.

In the two *Burkholderia* strains (both from a non-fungistatic soil), genes for synthesis of pyrrolnitrin, ACC deaminase, conversion of 2,3-butanediol to acetoin, acetoin catabolism and phosphate solubilization, were found. They also displayed genes for potential chitinases (Figure S2), and *Burkholderia GS-4* IT-111MI5 exhibited genes for beta-glucanases and genes of the AA10 family (Figure S3). *K. quasisacchari* IT-91MI3 (from fungistatic soil) had the complete set of genes for synthesis of 2,3-butanediol, acetoin and nitrogenase, as well as genes encoding for chitinases and especially cellulases.

Nine *Bacillota* strains were sequenced. Gene *alsSD* for acetoin biosynthesis was found in *B. licheniformis* IT-74MI3 (from fungistatic soil), *B. licheniformis* IT-13CA1 and *B. velezensis* IT-133MI5 (both from non-fungistatic soil), whereas gene *ydjL* for acetoin reductase/2,3-butanediol dehydrogenase was detected in *B. velezensis* IT-133MI5 and *Brevibacillus GS-3* IT-7CA2 (both from non-fungistatic soils). All the *Bacillota* strains presented genes encoding potential chitinases, up to 6 genes in *Brevibacillus GS-3* IT-7CA2 (from non-fungistatic soil) (Figure S2). *B. licheniformis* IT-74MI3 (from fungistatic soil), as well as *Brevibacillus GS-3* IT-7CA2, *B. licheniformis* IT-13CA1 and *B. velezensis* IT-133MI5 (from non-fungistatic soils) contained genes for AA10 family (Figure S3). Genes coding for beta-glucanases were detected only in *B. velezensis* IT-133MI5 (from non-fungistatic soil), while cellulase genes were found in four strains, especially in *B. licheniformis* IT-74MI3 (from fungistatic soil) and *B. licheniformis* IT-13CA1 (from non-fungistatic soil). Mannanase genes were detected in *B. licheniformis* IT-74MI3 (from fungistatic soil), and in *B*. *licheniformis* IT-13CA1 and *B. velezensis* IT-133MI5 (from non-fungistatic soils).

The *Bacteroidota Chryseobacterium GS-2* IT-36CA2 (from non-fungistatic soil) did not possess any of the genes investigated. Annotation of CAZymes showed that it harbored genes encoding potential chitinases (Figure S2), genes of the AA10 family (Figure S3) and genes coding for potential beta-glucanases, but it did not possess any genes involved in cellulase or mannanase production.

Putative biosynthetic gene clusters (BGCs), up to 20 in *B. ambifaria* IT-158MI4 and 19 in *B. velezensis* IT-133MI5 (from non-fungistatic soils), were identified in all strains (Table 6). The highest number of completed BGCs was 11 (in *B. velezensis* IT-133MI5). The highest number of BGCs in isolates from fungistatic soils was respectively 16 and 15 for *P. chlororaphis* IT-51CA3 and IT-162MI3, both with three completed BGCs.

In conclusion, isolates from both fungistatic and non-fungistatic soils possessed genes involved in biocontrol or plant-growth promotion. Distribution of phytobeneficial traits was taxa-specific to a large extent.

Table 6. Number of putative biosynthetic gene clusters (BGCs) and number of BGCs with completion 1 or 1*, in the 23 studied bacterial isolates, found using the antiSMASH (Blin et al., 2019), within the MicroScope platform.

Species name from TYGS	Bacterial isolate	Number of putative BGCs	Number of BGCs with completion 1	Number of BGCs with completion 1*
Isolates from fungistatic soils				
Bacillus licheniformis	IT-74MI3	12	3	0
Bacillus GS-1	IT-79MI2	13	2	0
Bacillus pseudomycoides	IT-19CA3	5	1	0
Bacillus pseudomycoides	IT-40CA3	2	1	0
Priestia megaterium	IT-180MI3	6	0	0
Priestia megaterium	IT-210MI2	7	0	0
Kosakonia quasisacchari	IT-91MI3	6	1	0
Pseudomonas donghuensis	IT-53CA3	5	0	0
Pseudomonas chlororaphis	IT-51CA3	16	1	2
Pseudomonas chlororaphis	IT-162MI3	15	1	2
Isolates from non-fungistatic soils				
Chryseobacterium GS-2	IT-36CA2	9	0	0
Brevibacillus GS-3	IT-7CA2	15	1	0
Bacillus licheniformis	IT-13CA1	12	3	0
Bacillus velezensis	IT-133MI5	19	11	0
Burkholderia GS-4	IT-111MI5	16	3	0
Burkholderia ambifaria	IT-158MI4	20	3	0
Pseudomonas soli	IT-47CA2	14	3	3
Pseudomonas chlororaphis	IT-48CA2	16	2	2
Pseudomonas brassicacearum	IT-43CA1	11	2	0
Pseudomonas GS-5	IT-194MI4	12	0	0
Pseudomonas GS-6	IT-196MI5	9	0	0
Pseudomonas GS-7	IT-93MI4	10	0	1
Pseudomonas GS-8	IT-232MI5	11	0	1

* When two or more genes in a single MIBiG (The Minimum Information about a Biosynthetic Gene cluster database) curated region were similar, the same gene in MicroScope database can hit on these MIBiG genes. When this happens, the completion can be higher than 1 (represented by 1*).

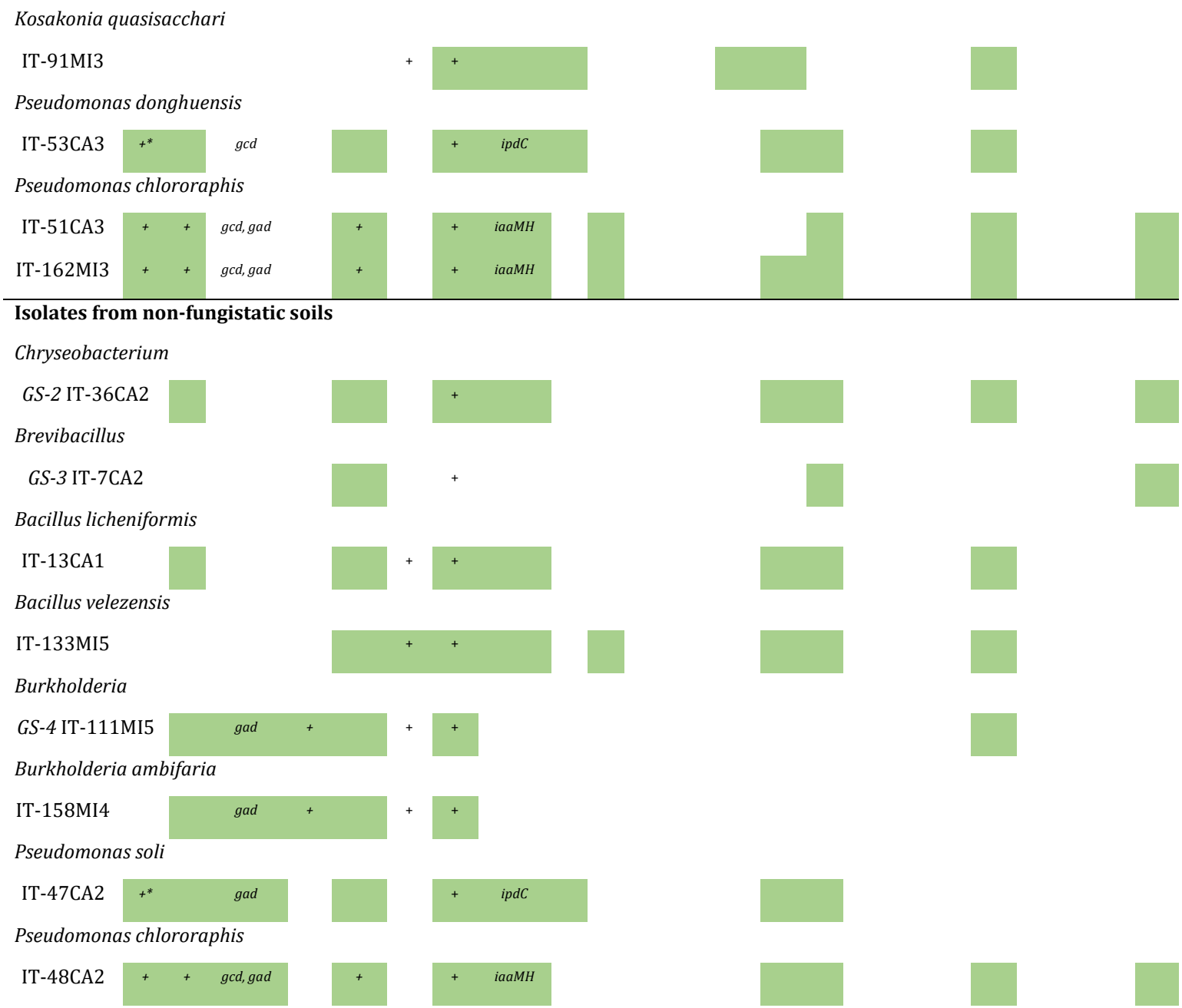
Correspondence between gene presence and *in vitro* activities

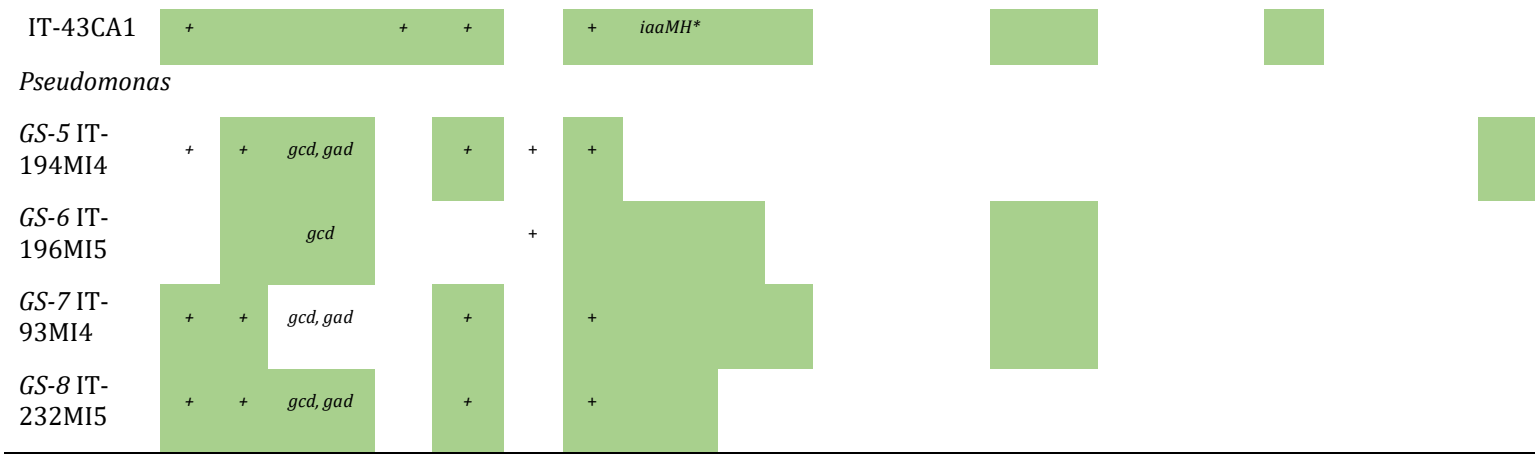
The production of HCN was confirmed *in vitro* in 8 of 9 *Pseudomonas* strains (from both fungistatic and non-fungistatic soils) carrying *hcnABC* genes (Table 7). Siderophore production was evidenced in all *Pseudomonas* strains, six of which (from both types of soils) carrying the *pvdL* pyoverdine gene, but the two *Bacillus* strains (from fungistatic or non-fungistatic soil), the two *Burkholderia* isolates and *Chryseobacterium* sp. IT-36CA2 (all three from non-fungistatic soils) produced siderophores despite lacking the *pvdL* gene. Phosphate solubilization was confirmed in strains originating only from non-fungistatic soils, i.e., six *Pseudomonas* strains and the two *Burkholderia* strains, but only some of them had glucose dehydrogenase gene *gad* and/or gluconate dehydrogenase gene *gcd*, while four *Pseudomonas* strains (three from fungistatic soils and one from non-fungistatic soil) had the genes but did not solubilize phosphate under the conditions tested. ACC deaminase activity was found in all *Burkholderia* strains and *P. brassicacearum* IT-43CA1 (all from non-fungistatic soils and carrying *acdS*). Protease activity was

detected in almost all strains (from both types of soils), including the seven *Pseudomonas* strains carrying the *aprA* gene. Cellulase activity was observed in only two fungistatic-soil strains (*B. licheniformis* IT-74MI3 and *P. megaterium* IT-210MI2) and one non-fungistatic-soil strain (*B. velezensis* IT-133MI5). Chitinase activity was confirmed *in vitro* for 20 of 23 strains with genes encoding chitinases (and/or genes of the AA10 family, as in *Pseudomonas GS-6* IT-196MI5) and coming from fungistatic or non-fungistatic soils. As many as 18 strains (from both types of soils) produced IAA, even though the *iaaMH* or *ipdC* genes were found in only six *Pseudomonas* strains. Production of indole-3-pyruvic acid was found in *B. pseudomycoides* IT-40CA3 and that of tryptophol in *Priestia megaterium* IT-180MI3 and *K. quasisacchari* IT-91MI3 (all three from fungistatic soils), and none of the strains produced indole-3-butyric acid, trans-zeatin riboside, kinetin, 6-benzylaminopurine, gibberellin A1, gibberellic acid or abscisic acid under the conditions tested. All the remaining phytohormones tested, i.e., indole-3-lactic acid, indole-3-carboxylic acid, indole-3-propionic acid, trans-zeatin, isopentenyl adenosine and kynurenic acid were produced by strains from both fungistatic and non-fungistatic soils. In summary, phosphate solubilization and ACC deaminase was recorded in strains from non-fungistatic soils, while production of indole-3-pyruvic acid and tryptophol was recorded only with strains originating from fungistatic soils, but the other traits tested were found in strains from fungistatic and non-fungistatic soils.

Table 7. Correspondence between gene presence and *in vitro* activities involved in plant-growth promotion and biocontrol in 23 isolates, according to the soil fungistasis status. Activity is marked with a green colour. Gene corresponding to a given activity *in vitro* (when found in the genomes) is indicated with + (the whole gene cluster), and when for certain property there are several possible pathways to achieve a function, names of the genes found in the genome are indicated. Cellulase and chitinase were predicted using dbCAN2 (v.3; Zhang et al., 2018) and compared with the CAZy database using HMMER (v.3.3; Eddy, 2011). Prediction of function and substrate specificity of CAZyme families or subfamilies was performed based on a review of activities assigned to CAZymes with known structures (characterized enzymes) in the CAZy database (http://www.cazy.org) (Lombard et al., 2014) and manually curated, as previously described (López-Mondéjar et al., 2022).

Bacterial isolate	HCN	Siderophores	Phosphate solubilization	ACC deaminase	Protease	Cellulase	Chitinase	Indole-3-acetic acid	Indole-3-lactic acid	Indole-3-carboxylic acid	Indole-3-pyruvic acid	Indole-3-butyric acid	Tryptophol	Indole-3-propionic acid	Trans-zeatin	Trans-zeatin riboside	Kinetin	6-Benzylaminopurine	Isopentenyl adenosine	Gibberellin A1	Gibberellic acid	Abscisic acid	Kynurenic acid
Isolates from fungistatic soils																							
Bacillus licheniformis																							
IT-74MI3						+	+																
Bacillus																							
GS-1 IT-79MI2						+	+																
Bacillus pseudomycoides																							
IT-19CA3							+																
IT-40CA3							+																
Priestia megaterium (formerly *Bacillus megaterium*)																							
IT-180MI3							+																
IT-210MI2							+																


Kosakonia quasisacchari
IT-91MI3
+
+
Pseudomonas donghuensis
IT-53CA3
+*
gcd
+
ipdC
Pseudomonas chlororaphis
IT-51CA3
+
+
gcd, gad
+
+
iaaMH
IT-162MI3
+
+
gcd, gad
+
+
iaaMH
Isolates from non-fungistatic soils
Chryseobacterium
GS-2 IT-36CA2
+
Brevibacillus
GS-3 IT-7CA2
+
Bacillus licheniformis
IT-13CA1
+
+
Bacillus velezensis
IT-133MI5
+
+
Burkholderia
GS-4 IT-111MI5
gad
+
+
+
Burkholderia ambifaria
IT-158MI4
gad
+
+
+
Pseudomonas soli
IT-47CA2
+*
gad
+
ipdC
Pseudomonas chlororaphis
IT-48CA2
+
+
gcd, gad
+
+
iaaMH



*_hcnA_* found with <70 % identity (63 % for isolate IT-47CA2 and 69 % for isolate IT-53CA3); *_iaaH_* found with only 33 % identity in isolate IT-43CA1

Inhibitory effect of bacterial volatile organic compounds on fungal growth and inhibitory effect of bacterial exudates on sporulation of *Fusarium graminearum*

Mycelial growth of *F. graminearum* Fg1 was inhibited by VOCs of only four antagonistic strains, i.e., *P. soli* IT-47CA2 (by 47.1%), *Pseudomonas GS-5* IT-194MI4 (by 23.5%), and *Burkholderia ambifaria* IT-158MI4 (by 41.2%) and *Burkholderia GS-4* IT-111MI5 (by 11.8%) (Figure 1). All four originated from non-fungistatic soils.

In a microplate assay, conidia germination of *F. graminearum* Fg1 was inhibited by exudates of *P. donghuensis* IT-53CA3 (from fungistatic soil) by 75 %, and *Burkholderia GS-4* IT-111MI5 (from non-fungistatic soil) by 26.6 %. Other strains (from fungistatic or non-fungistatic soils), i.e., *Chryseobacterium GS-2* IT-36CA2, *Brevibacillus GS-3* IT-7CA2, *B. licheniformis* IT-74MI3, *B. licheniformis* IT-13CA1, *Bacillus GS-1* IT-79MI2, *B. pseudomycoides* IT-19CA3 and IT-40CA3, *Priestia megaterium* IT-210MI2 and *Burkholderia ambifaria* IT-158MI4 also inhibited conidia germination, but at levels < 20% (Figure 1).

In summary, VOCs of certain antagonistic strains (from non-fungistatic soils only) affected mycelial growth of *F. graminearum* Fg1. In contrast, exudates of several strains from fungistatic or non-fungistatic soils inhibited conidia germination.

Additional genomic analyses of the most promising antagonistic strains

The most promising antagonistic bacteria, i.e., those that produced VOCs inhibiting fungal mycelial growth and/or whose exudates inhibited conidia germination (Figure 1) were chosen for further analysis. They included *Brevibacillus GS-3* IT-7CA2, *B. velezensis* IT-133MI5, *Burkholderia ambifaria* IT-158MI4, *P. soli* IT-47CA2, *P. chlororaphis* IT-48CA2 and *Pseudomonas GS-5* IT-194MI4 from non-fungistatic-soils, but only *P. donghuensis* IT-53CA3 from fungistatic soil. On one hand, we analyzed further their genomic plant-beneficial traits. On the other hand, we performed plant assay in the presence of *F. graminearum* Fg1.

BGCs analysis showed that *Brevibacillus* sp. *GS-3* IT-7CA2 harbored gene clusters coding for antibiotics, such as edeine, tyrocidin (surfactin), lipopeptide antibiotic, linear gramicidin, bacillaene-like antifungal product, and siderophores (Table S2). In the genome of *B. velezensis* strain IT-133MI5, BGCs were found for the production of antibiotics (mycosubtilin, macrolactin, plipastatin, difficidin, mersacidin, surfactin, bacilysin, lanthipeptides and bacillibactin), while *B. ambifaria* IT-158MI4 had the potential of producing phenazine-like compound, pyrrolnitrin (as confirmed by BLAST), non-ribosomal antifungal oligopeptides, as well as the siderophores enterobactin and ornibactin. *P. chlororaphis* IT-48CA2 possessed genes for phenazine, pyrrolnitrin (as confirmed by BLAST), putative bacitracin/enterobactin, mangotoxin and different siderophores. *P. donghuensis* IT-53CA3 had BGCs for pyoverdine and mangotoxin biosynthesis, while *P. soli* IT-47CA2 had BGCs for production of cyclic lipopeptide xantholysin, dapdiamides, mangotoxin and siderophores. Finally, *Pseudomonas GS-5* IT-194MI4 had potential of producing mangotoxin and different siderophores. In summary, the genomes of these seven strains displayed BGCs putatively coding for production of siderophores and antibiotics, potentially involved in biocontrol.

Effects of selected antagonistic bacteria on wheat phytoprotection from *Fusarium graminearum*

In the plant assay, the addition of *F. graminearum* Fg1 alone resulted in a significantly lower number of germinated seeds at 14 days (Figure 2A), high disease symptoms (Figure 2B), lower biomass (Figure 2C) and lower chlorophyll rate at 45 days (Figure 2D), in comparison with non-inoculated seeds. In comparison with seeds inoculated with *F. graminearum* Fg1, there was a trend for higher number of germinated seeds when inoculation was carried out with 3 of 7 bacteria, i.e., *B. ambifaria* IT-158MI4 and *Pseudomonas GS-5* IT-194MI4 (trend significant at $P < 0.05$) from non-fungistatic soil, and *P. donghuensis* IT-53CA3 from fungistatic soil. In addition, bacterial inoculation resulted in lower disease symptoms with *B. velezensis* IT-133MI5, *P. soli* IT-47CA2, *P. chlororaphis* IT-48CA2, *Pseudomonas* sp. *GS-5* IT-194MI4 (all from non-fungistatic soils) and *P. donghuensis* IT-53CA3 (from fungistatic soil). Finally, biomass was lower with *B. ambifaria* IT-158MI4 and the four *Pseudomonas* strains, and chlorophyll rate of germinated plants was lower with *B. ambifaria* IT-158MI4 and *Pseudomonas* sp. *GS-5* IT-194MI4.

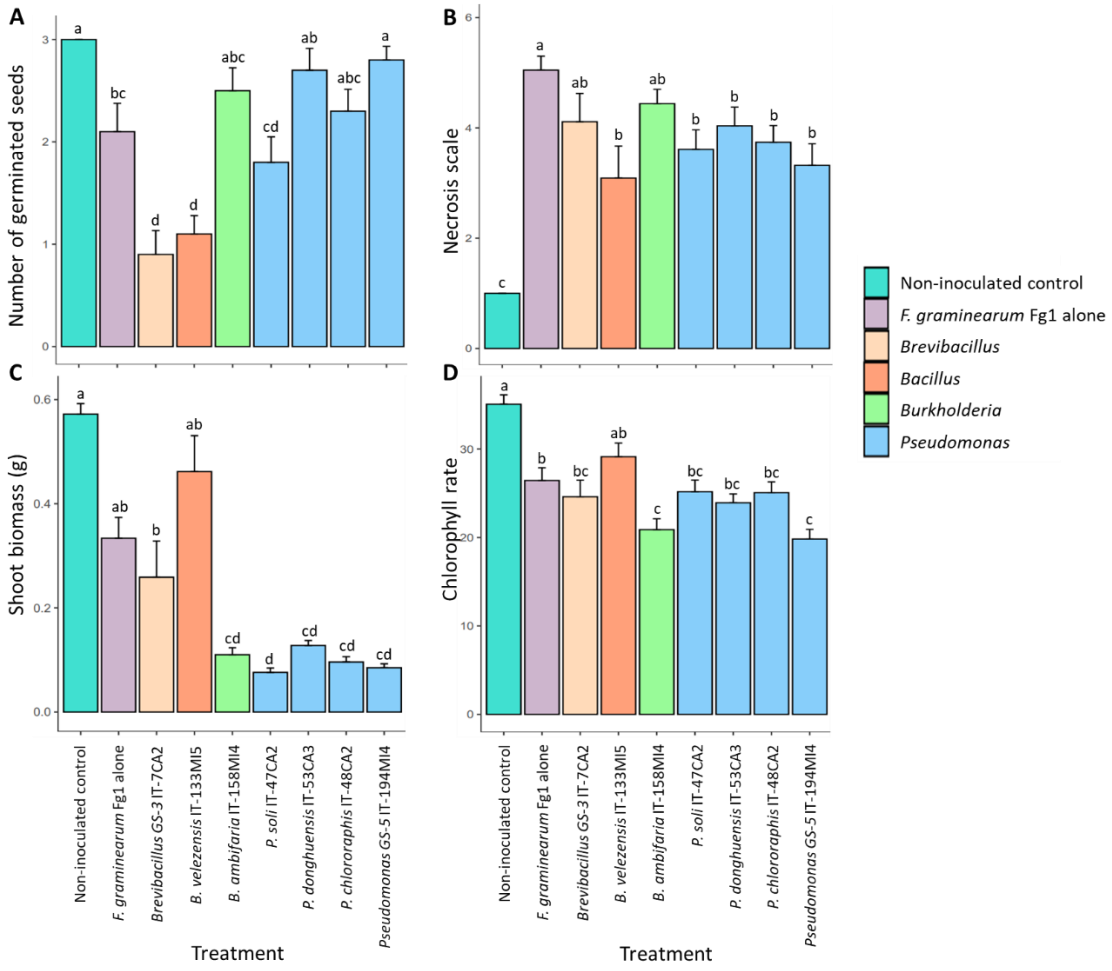


Figure 2. Results of the *in planta* protection assay. (A) Number of germinated seeds at 2 weeks after inoculation with antagonistic bacteria and *Fusarium graminearum* Fg1. Results are presented as mean + standard error (n = 10). Data were treated with Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with letters a to d. (B) Disease symptoms of

crown-rot at 2 weeks after inoculation with antagonistic bacteria and *Fusarium graminearum* Fg1. Non-germinated plants were regarded as missing data (NA). Results are presented as mean + standard error (n = 10). Data were treated with Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with significance letters a to c. (C) Shoot biomass of wheat plants at 2 weeks after inoculation with antagonistic bacteria and *Fusarium graminearum* Fg1. Non-germinated plants were regarded as missing data (NA). Results are presented as mean + standard error (n = 10). Data were treated with Kruskal-Wallis and Dunn's test ($P < 0.05$). Statistical differences are shown with letters a to d. (D) Chlorophyll rate of wheat plants at 2 weeks after inoculation with antagonistic bacteria and *Fusarium graminearum* Fg1. The chlorophyll rate of each wheat plant was the average of three measurements, taken on the 5th, 6th and 7th grown leaf. Non-germinated plants and plants without grown leaves were regarded as missing data (NA). Results are presented as mean + standard error (n = 10). Data were treated with ANOVA and Tukey's test ($P < 0.05$), and statistical differences are shown with letters a to c.

In summary, *Pseudomonas GS-5* IT-194MI4 (from non-fungistatic soil) enhanced wheat germination and conferred protection from crown-rot disease, but at the expense of shoot biomass and chlorophyll rate. The three other *Pseudomonas* strains and *B. ambifaria* IT-158MI4 conferred some protection but without improving seed germination, and they affected shoot biomass.

Discussion

The ascomycete *F. graminearum* causes wilting and necrosis of many economically important plant species, including wheat (Ma et al., 2013), and it is suggested that this fungus might cause increasing damage to agricultural crops in the ongoing climate change context (Vaughan et al., 2016). The rhizosphere represents a source of microorganisms that may control *Fusarium* (Wang et al., 2015; Jangir et al., 2018), especially if biocontrol strains are sought in disease-suppressive soils (Weller et al., 2007). Against this background, fungistatic soils have been neglected to supply plant-protecting microorganisms, including for *Fusarium* diseases (Stutz et al., 1986; Lemanceau & Alabouvette, 1991; Fuchs et al., 1997; Raaijmakers & Weller, 1998). In fungistatic soils, general soil suppressiveness is conferred via a range of competitive and other interactions between the soil microbiota and pathogen (Garbeva et al., 2011; de Boer et al., 2019).

Here, our screening of antagonistic isolates from fungistatic (soils MI2, MI3 and CA3) and non-fungistatic Serbian soils (soils MI4, MI5, CA1 and CA2) selected *Brevibacillus GS-3* IT-7CA2, *Bacillus velezensis* IT-133MI5, *Burkholderia ambifaria* IT-158MI4, *Pseudomonas soli* IT-47CA2, *Pseudomonas chlororaphis* IT-48CA2, *Pseudomonas GS-5* IT-194MI4 (all from non-fungistatic soils), and *Pseudomonas donghuensis* IT-53CA3 (from fungistatic soil). Therefore, contrarily to our hypothesis, fungistasis did not prove to be an over-riding factor favoring the identification of bacteria with biocontrol potential.

B. velezensis IT-133MI5 and the four *Pseudomonas* strains (including *P. donghuensis* IT-53CA3 from fungistatic soil) limited disease symptoms in wheat. In addition, *B. velezensis* IT-133MI5 inhibited seed germination, but antagonism in a dual-culture assay does not necessarily mean good performance in plants (Besset-Manzoni et al., 2019). The four *Pseudomonas* resulted in reduced shoot biomass and *Pseudomonas GS-5* IT-194MI4 also gave reduced chlorophyll rate (but it improved seed germination), pointing to a trade-off between plant protection and plant growth (Karasov et al., 2017). *Pseudomonas* can induce systemic resistance (ISR) in plants through production of siderophores (Bakker et al., 2007), which are synthesized by all four of

them. ISR can also be triggered by different VOCs, such as acetoin and 2,3-butanediol (Ryu et al., 2004; Kerečki et al., 2022), whose biosynthetic genes were found in *B. velezensis* IT-133MI5. Genome sequencing also identified traits that can lead to direct *Fusarium* inhibition. Thus, *P. chlororaphis* IT-48CA2 harbored genes involved in production of HPR, phenazine and pyrrolnitrin. All four *Pseudomonas* had genes for HCN, which has antifungal effects (Ramette et al., 2003), and HCN production was confirmed for all of them but *Pseudomonas GS-5* IT-194MI4. Protease production *in vitro* was observed in all four *Pseudomonas* and *B. velezensis* IT-133MI5; *P. chlororaphis* IT-48CA2 and *Pseudomonas GS-5* IT-194MI4 had *aprA* genes for alkaline metalloproteinase production. Chitinase was produced by all four *Pseudomonas* and *B. velezensis* IT-133MI5, while cellulase was produced only by *B. velezensis* IT-133MI5. Lytic enzymes, such as proteases, cellulases and chitinases play a key role in antagonism towards *Fusarium* (Rathore et al., 2020).

Different selective and non-selective isolation media were used, and dual-culture assay (a common screening procedure when looking for potential biocontrol agents; Paulitz et al., 1992; Besset-Manzoni et al., 2019) with the 244 isolates gave 12 isolates that inhibited *F. graminearum* Fg1 growth by more than 50% and 11 other isolates altering fungal morphology. Altogether, we obtained similar numbers of strains from both types of soils, i.e., 10 from fungistatic and 13 from non-fungistatic soils (Figure 1). Therefore, the loss of antagonistic isolates from fungistatic soils took place with the second screening, which focused on the effect of VOCs on Fg1 growth (significant only with isolates from non-fungistatic soils) and the impact of bacterial exudates on conidia germination (significant isolates from fungistatic and non-fungistatic soils). Some antagonistic isolates from non-fungistatic soils can indeed produce VOCs, as *Pseudomonas* strain IT-47CA2 releases HCN *in vitro*, *Pseudomonas* strain IT-194MI4 possesses genes for HCN production, whereas *Burkholderia* strains IT-158MI4 and IT-111MI5 harbor *adh* gene involved in 2,3-butanediol conversion to acetoin. *Pseudomonas* and *Burkholderia* species can be effective at inhibiting *Fusarium* mycelia development through production of different VOCs (Cordero et al., 2014; Weisskopf, 2014).

Whole genome sequencing of the 23 antagonistic bacteria showed that they belong to seven genera, i.e., *Pseudomonas* (10 strains), *Bacillus* (6 strains), *Priestia* (formerly within *Bacillus*; 2 strains), *Brevibacillus* (1 strains), *Burkholderia* (2 strains), *Kosakonia* (1 strain) and *Chryseobacterium* (1 strain). As expected, representatives from the genera *Pseudomonas* and *Bacillus* were the most abundant in our collection, in line with previous screening studies (Janssen, 2006; Prashar et al., 2014; Nwachukwu et al., 2021). It was the case in both fungistatic and non-fungistatic soils, indicating that there was not a major taxonomic bias in the procedure. Surprisingly, none of the isolates from the SAA media, i.e., from the phylum *Actinomycetota* (formerly *Actinomycetes*) could inhibit *F. graminearum* Fg1 on plate, although this phylum is known for production of antibiotics (Cuesta et al., 2012) and lytic enzymes (Soltanzadeh et al., 2016). Previous research for biocontrol agents against *Fusarium* rarely included *Brevibacillus* (Johnson et al., 2020), *Burkholderia* (Ho et al., 2015), *Chryseobacterium* (Khan et al., 2006) and *Kosakonia* (formerly *Enterobacter*) (Tsuda et al., 2001), however in current study they did not provide phytoprotection.

Whole genome sequencing is a useful approach to probe the genetic potential of promising isolates, so this should become standard procedure during analysis of potential biocontrol agents (Cai et al., 2017; Nelkner et al., 2019). Here, it evidenced (Table 5) that *P. chlororaphis* strains IT-51CA3 and IT-162MI3 (from fungistatic soils) harbored genes that encode antifungal metabolites, such as phenazine, HPR, pyrrolnitrin and HCN, as did *P. chlororaphis* IT-48CA2 (from non-fungistatic soil), pointing that these properties are related to taxonomy, as

already documented in this species (Calderón et al., 2013; Loewen et al., 2014). Only *P. brassicacearum* IT-43CA1 (from non-fungistatic soils) harbored genes for DAPG production, a key biocontrol property occurring in *P. brassicacearum* and other *Pseudomonas* species (Almario et al., 2017). In *Brevibacillus GS-3* IT-7CA2, *B. licheniformis* IT-13CA1 (from non-fungistatic soil) and *B. licheniformis* IT-74MI3 (from fungistatic soil), we found only genes involved in acetoin and 2,3-butanediol metabolism. In *Burkholderia ambifaria* IT-158MI4, genes involved in pyrrolnitrin production were evidenced but this strain did not provide plant protection in the greenhouse.

Whole genome sequencing is also useful to clarify the taxonomic status and general ecology of promising isolates, as illustrated by the identification of as many as 8 novel genomospecies from 5 genera (including well-studied *Pseudomonas*) from the 23 antagonistic strains. Therefore, further research is needed to formally describe these 8 potentially-new species.

In conclusion, both fungistatic and non-fungistatic soils can be sources of antagonistic bacteria, but fungistatic soils were not of outstanding value when screening further, especially based on VOCs inhibition of mycelial growth. Whole-genome sequencing was useful to gain key insight into the biocontrol potential and taxonomic status of antagonistic strains.

SEQUENCE ACCESSION NUMBERS

Whole genome sequences (raw and assembled) are deposited at the EBI/EMBL database under the accession number PRJEB59762.

FUNDING

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICAL STATEMENT

The experiments did not involve human participants and/or animals.

SUPPLEMENTARY DATA

Table S1. Accession numbers of genome protein sequences searched for in the bacterial genomes, involved in biocontrol and plant-growth promotion.

Gene	Accession number	Reference species
darA	AAN18031.1	*Pseudomonas chlororaphis* subsp. *aurantiaca*
darB	AAN18032.1	*Pseudomonas chlororaphis* subsp. *aurantiaca*
darC	AAN18033.1	*Pseudomonas chlororaphis* subsp. *aurantiaca*
phzA	AAC18900.1	*Pseudomonas fluorescens* 2-79
phzB	AAC18901.1	*Pseudomonas fluorescens* 2-79
phzC	AAC18902.1	*Pseudomonas fluorescens* 2-79
phzD	AAC18903.1	*Pseudomonas fluorescens* 2-79
phzE	AAC18904.1	*Pseudomonas fluorescens* 2-79
phzF	AAC18905.1	*Pseudomonas fluorescens* 2-79
phzG	AAC18906.1	*Pseudomonas fluorescens* 2-79
pchA	PFLCHA0_c35300	*Pseudomonas protegens* CHA0
pchB	PFLCHA0AM_3611	*Pseudomonas protegens* CHA0
pchC	PFLCHA0_c35310	*Pseudomonas protegens* CHA0
pchD	PFLCHA0_c35370	*Pseudomonas protegens* CHA0
pchE	PFLCHA0_c35340	*Pseudomonas protegens* CHA0
pchF	PFLCHA0_c35330	*Pseudomonas protegens* CHA0
phlA	PSF113_2462	*Pseudomonas fluorescens* F113
phlB	PSF113_2460	*Pseudomonas fluorescens* F113
phlC	PSF113_2461	*Pseudomonas fluorescens* F113
phlD	PSF113_2459	*Pseudomonas fluorescens* F113
prnA	PFLCHA0_c36450	*Pseudomonas protegens* CHA0
prnB	PFLCHA0_c36460	*Pseudomonas protegens* CHA0
prnC	PFLCHA0_c36470	*Pseudomonas protegens* CHA0
prnD	PFLCHA0_c36480	*Pseudomonas protegens* CHA0
hcnA	PSF113_2367	*Pseudomonas fluorescens* F113
hcnB	PSF113_2368	*Pseudomonas fluorescens* F113
hcnC	PSF113_2369	*Pseudomonas fluorescens* F113
pltA	PFLCHA0_c28450	*Pseudomonas protegens* CHA0

pltB	PFLCHA0_c28460	*Pseudomonas protegens* CHA0
pltC	PFLCHA0_c28470	*Pseudomonas protegens* CHA0
pltD	PFLCHA0_c28480	*Pseudomonas protegens* CHA0
pltE	PFLCHA0_c28490	*Pseudomonas protegens* CHA0
pltF	PFLCHA0_c28500	*Pseudomonas protegens* CHA0
pltG	PFLCHA0_c28510	*Pseudomonas protegens* CHA0
pltL	PFLCHA0_c28440	*Pseudomonas protegens* CHA0
pltM	VAV69124.1	*Pseudomonas protegens* CHA0
pvdL	VAV70531.1	*Pseudomonas protegens* CHA0
pmsC	CAA70528.1	*Pseudomonas fluorescens*
pmsE	CAA70529.1	*Pseudomonas fluorescens*
pmsA	CAA70530.1	*Pseudomonas fluorescens*
pmsB	CAA70531.1	*Pseudomonas fluorescens*
fitD	AGL84796.1	*Pseudomonas protegens* CHA0
acdS	PSF113_3500	*Pseudomonas fluorescens* F113
iacA	ABY62757.1	*Pseudomonas putida*
iacB	ABY62758.1	*Pseudomonas putida*
iacC	ABY62759.1	*Pseudomonas putida*
iacD	ABY62760.1	*Pseudomonas putida*
iacE	ABY62761.1	*Pseudomonas putida*
iacF	ABY62762.1	*Pseudomonas putida*
iacG	ABY62763.1	*Pseudomonas putida*
iacH	ABY62765.1	*Pseudomonas putida*
iacI	ABY62766.1	*Pseudomonas putida*
acoA	PFLCHA0_c22170	*Pseudomonas protegens* CHA0
acoB	PFLCHA0_c22180	*Pseudomonas protegens* CHA0
acoC	PFLCHA0_c22190	*Pseudomonas protegens* CHA0
acoX	PFLCHA0_c22160	*Pseudomonas protegens* CHA0
bdhA	JV497_v1_130113	*Pseudomonas* sp. JV497
adh	AAB58982.1	*Pseudomonas putida*
budA	AAA25054.1	*Raoultella terrigena*
budB	AAA25055.1	*Raoultella terrigena*

budC	AAA25056.1	*Raoultella terrigena*
alsS	AAA22222.1	*Bacillus subtilis*
alsD	AAA22223.1	*Bacillus subtilis*
ilvB	AAB81919.1	*Lactococcus lactis* subsp. *lactis* NCDO 2118
ilvN	AAB81920.1	*Lactococcus lactis* subsp. *lactis* NCDO 2118
ydjL	ALH44116.1	*Bacillus subtilis* subsp. *subtilis*
gcd	NP_250980.1	*Pseudomonas aeruginosa* PAO1
gad	NP_250955.1	*Pseudomonas aeruginosa* PAO1
nifH	AAD03796.1	*Trichodesmium erythraeum* IMS101
nifD	AAD03797.1	*Trichodesmium erythraeum* IMS101
nifK	AAD03798.1	*Trichodesmium erythraeum* IMS101
nirK	PFLCHA0_c54550	*Pseudomonas protegens* CHA0
nirS	AAG34381.1	*Pseudomonas fluorescens*
aprA	PFLCHA0_c32400	*Pseudomonas protegens* CHA0
ipdC	PP_2552	*Pseudomonas putida* KT2440
ppdC	WP_011158661.1	*Rhodopseudomonas palustris*
iaaM	PSF113_5381	*Pseudomonas fluorescens* F113
iaaH	JV395A_v1_10784	*Pseudomonas* sp. JV395A
efe	BBI44544.1	*Pseudomonas syringae* pv. *actinidiae*

Table S2. List of secondary metabolites identified using the antiSMASH (Blin et al., 2019) and manually curated in *Brevibacillus GS-3* IT-7CA2, *Bacillus velezensis* IT-133MI5, *Burkholderia ambifaria* IT-158MI4, *Pseudomonas chlororaphis* IT-48CA2, *Pseudomonas donghuensis* IT-53CA3, *Pseudomonas soli* IT-47CA2 and *Pseudomonas GS-5* IT-194MI4. The start, end, length and region type of the predicted biosynthetic gene cluster are shown. Abbreviations: transAT-PKS (trans-acyltransferase polyketide synthases), NRPS (non-ribosomal peptide synthetases), T3PKS (type III polyketide synthases), LAP (linear azol(in)e-containing peptides), T1PKS (type I polyketide synthases), hserlactone (homoserine lactone), PpyS-KS (PPY-like pyrone) and NAGGN (N-acetylglutaminylglutamine amides).

Isolate	Start	End	Length	Region type	Pathway manually curated
Brevibacillus GS-3 IT-7CA2	49394	126050	76657	transAT-PKS, NRPS	Edeine
	145711	204801	59091	NRPS, transAT-PKS-like	Subtilisin-like alkaline serine protease
	453835	494899	41065	T3PKS	Spore germination factor
	602938	624920	21983	terpene	Lipopolysacharide synthesis, terpene synthesis, sporulation related
	759927	867046	107120	NRPS, transAT-PKS-like, transAT-PKS	Bacillaene like, natural product with antifungal properties
	1389577	1413147	23571	LAP, bacteriocin	Maturation of compound from a ribosomally produced precursor polypeptide
	1522244	1591382	69139	NRPS	Tyrocidin (surfactin) synthesis
	2279275	2346852	67578	NRPS	Lipopeptide antibiotic synthesis
	2926082	2936930	10849	bacteriocin	Encapsulins
	3197643	3258150	60508	NRPS	Siderophore or antibiotic
	4892513	4915134	22622	lanthipeptide	Lanthipeptide involved in spore germination
	4989239	5042547	53309	NRPS	Anabaenopeptin NZ 857 / nostamide A
	5119171	5187480	68310	lanthipeptide, NRPS	Linear gramicidin synthetase
	5361405	5375121	13717	siderophore	Petrobactin siderophore
	6316909	6319581	2673	NRPS	Unknown
B. velezensis IT-133MI5	1	79202	79202	betalactone, NRPS, transAT-PKS	Mycosubtilin
	143184	252423	109240	transAT-PKS-like, transAT-PKS, NRPS, T3PKS	Bacillaene/alkaline serine protease aprX
	474237	562458	88222	transAT-PKS	Macrolactin
	865867	886607	20741	terpene	Unknown
	969936	1011180	41245	PKS-like	Polyketide
	1075641	1113576	37936	NRPS	
	3824989	3834225	9237	NRPS	Plipastatin
	3834326	3842245	7920	NRPS	
	1138866	1160749	21884	terpene	Sesquarterpenes
	1222008	1263108	41101	T3PKS	Antibiotic
	1378699	1484878	106180	transAT-PKS-like, transAT-PKS	Difficidin
	2319521	2342709	23189	lanthipeptide	Mersacidin
	2694377	2719769	25393	NRPS	
	3815603	3824888	9286	NRPS	Surfactin
	2941791	2969557	27767	NRPS	
	2738042	2779460	41419	other	Bacilysin synthesis
	3068358	3090973	22616	lanthipeptide	Lanthipeptide
	3336546	3374388	37843	NRPS	Bacillibactin
	3484796	3495134	10339	bacteriocin	Circular bacteriocins, antimicrobial

					peptides
B. ambifaria IT-158MI4	246251	293810	47560	T1PKS	Putative heparinase II/III family protein
	782932	827843	44912	T1PKS, NRPS-like	Unknown
	1195618	1216259	20642	hserlactone	Acyl-homoserine-lactone synthase, involved in quorum sensing
	1361820	1408642	46823	NRPS	Enterobactin like siderophore
	1623867	1644856	20990	terpene	Unknown
	2304168	2345853	41686	phosphonate	Unknown
	2380170	2425048	44879	arylpolyene	Unknown
	2480912	2501976	21065	terpene	Non-heme iron decarboxylase, involved in antibiotic novobiocin synthesis
	2707158	2748381	41224	arylpolyene	Cardiolipin synthase C
	3554068	3574496	20429	phenazine	Phenazine like compound
	3934141	3944956	10816	bacteriocin	Nanocompartment encapsulin Linocin M18
	4441363	4485247	43885	T1PKS	Adhesin bpaC, virulence factor, biofilm formation
	4643983	4654369	10387	ectoine	Partial ectoine synthesis pathway
	6250299	6260697	10399	ectoine	Partial ectoine synthesis pathway
	4708504	4732599	24096	terpene	Squalene biosynthesis
	4918184	4972895	54712	NRPS	Siderophore ornibactin synthesis
	5778732	5819817	41086	other	Pyrrolnitrin
	5941856	5963904	22049	terpene	Unknown
	6000086	6020691	20606	hserlactone	Unknown lipopeptides
	6555814	6641248	85435	NRPS, T1PKS	Non-ribosomal oligopeptides with antifungal activity
P. chlororaphis IT-48CA2	409268	428234	18967	siderophore	Polycarboxylate siderophore staphyloferrin B, IucA/IucC
	648898	675789	26892	terpene	Beta-caryophyllene-like sesquiterpenoid
	967567	1011187	43621	arylpolyene	Lipoprotein
	1778015	1809062	31048	NRPS	Pyoverdine synthesis
	1997392	2020639	23248	betalactone	Biotin synthesis pathway, fatty acid (long saturated)
	3632874	3643770	10897	bacteriocin	Unknown
	4180078	4190971	10894	bacteriocin	Unknown lipoprotein
	4613843	4636461	22619	phenazine, hserlactone	Phenazine
	4691104	4711784	20681	hserlactone	Quorum sensing involved pathway 2 *psy*
	5140419	5161078	20660	hserlactone	Quorum sensing involved pathway 1 *rhl*
	5313688	5372696	59009	NRPS	Putative bacitracin/enterobactin
	5885184	5938200	53017	NRPS	Pyoverdine synthetase
	6073140	6119367	46228	NRPS, resorcinol	Pyoverdine synthetase
	6689686	6720468	30783	NRPS-like	Mangotoxin biosynthesis
	6720569	6749771	29203	other	Pyrrolnitrin
	6790107	6793135	3029	NRPS	Massetolide, orfamide, syringopeptin like synthesis
P. donghuensis IT-53CA3	1138554	1206048	67495	NRPS	Pyoverdine
	3059651	3112613	52963	NRPS	Pyoverdine
	3550188	3593025	42838	NRPS-like	Secondary metabolite
	5009796	5053409	43614	arylpolyene	Lipoprotein
	5583820	5605556	21737	NRPS-like	Mangotoxin biosynthesis
P. soli IT-47CA2	1814889	1842944	28056	NRPS	
	1843049	1880909	37861	NRPS	Xantholysin (cyclic lipopeptides)
	2525370	2598975	73606	NRPS	
	2012633	2078210	65578	NRPS	Pseudomonine heterocyclic siderophore

	2201021	2242208	41188	T3PKS	Mevalonate pathway isoprenoide synthesis
	2278901	2331848	52948	NRPS	Pyoverdine synthetase A
	2495147	2516196	21050	PpyS-KS	Pseudopyronines A and B
	2701819	2712640	10822	bacteriocin	Unknown
	4017294	4049445	32152	NRPS	Lipopeptide siderophores
	5549990	5559838	9849	NAGGN	Dapdiamides, tripeptide antibiotics
	5611199	5626725	15527	NRPS-like	Mangotoxin biosynthesis
	5654779	5666507	11729	NRPS	
	5666608	5672935	6328	NRPS	Siderophore
	5681332	5684101	2770	NRPS	
Pseudomonas GS-5 IT-194MI4	23130	36540	13411	butyrolactone	Unknown
	517198	528034	10837	bacteriocin	Cellular processes, signalling transduction
	2172527	2181555	9029	bacteriocin	Unknown
	2209373	2221343	11971	bacteriocin	Unknown
	2376537	2388459	11923	siderophore	Unknown
	2984871	3061145	76275	NRPS	Pyoverdine synthesis
	3186077	3213173	27097	betalactone	Biotin like synthesis pathway, fatty acid (long saturated)
	3511162	3564145	52984	NRPS	Pyoverdine synthetase A
	3719392	3734144	14753	NAGGN	N-acetylglutaminylglutamine synthetase/cell wall synthesis
	3932490	3976095	43606	arylpolyene	Lipoprotein
	5315442	5326287	10846	bacteriocin	Cardiolipin synthase A
	6372519	6389175	16657	NRPS-like	Mangotoxin biosynthesis

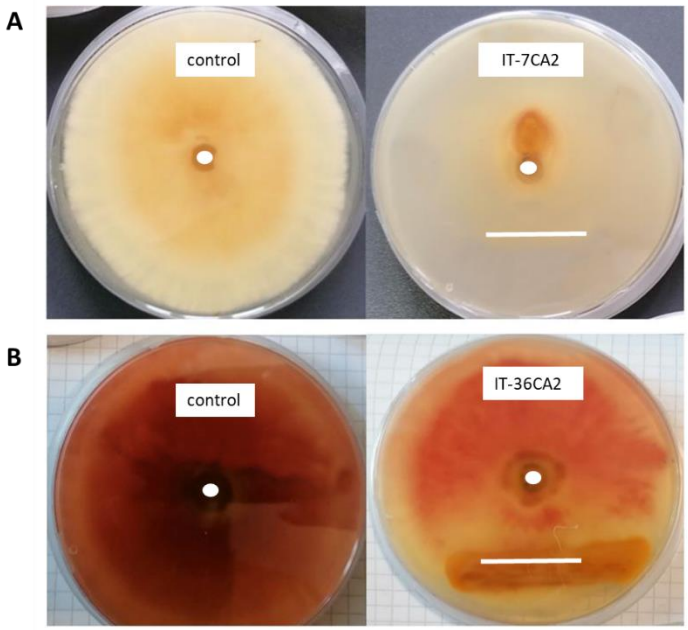

Figure S1. Antagonistic activity of bacterial isolates *Brevibacillus GS-3* IT-7CA2 (A) and *Chryseobacterium GS-2* IT-36CA2 (B) towards *Fusarium graminearum* Fg1 in a dual-culture assay. Left: control plate with *Fusarium graminearum* Fg1. Right: plate with *Fusarium graminearum* Fg1 and the bacterial isolate. The white dot on Petri dish represents the place of inoculation of *Fusarium graminearum* Fg1 and the white line represents the point of bacterial inoculation.

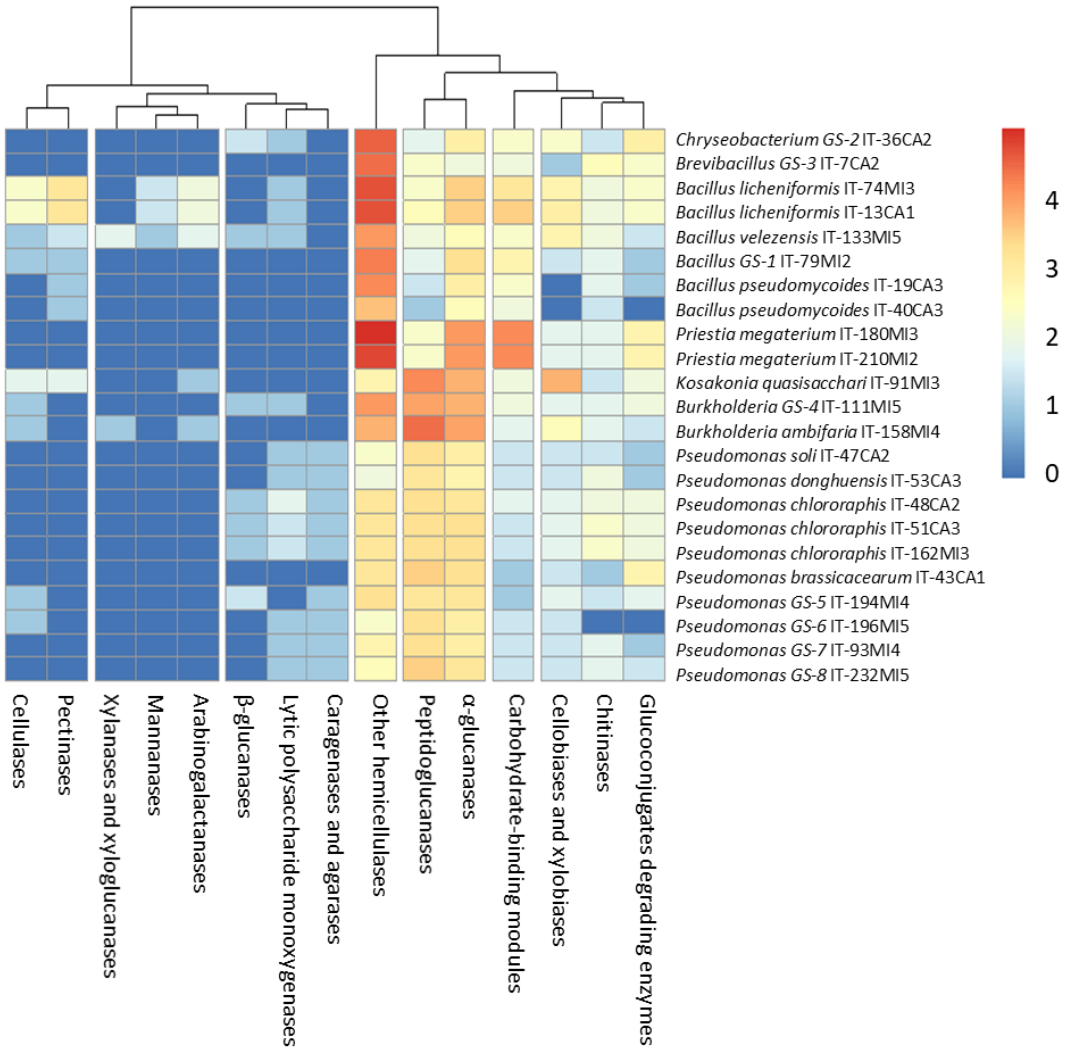

Figure S2. Heatmap showing the abundance of CAZyme genes annotated for each function found in the genomes of the 23 bacteria. Legend shows transformed counts.

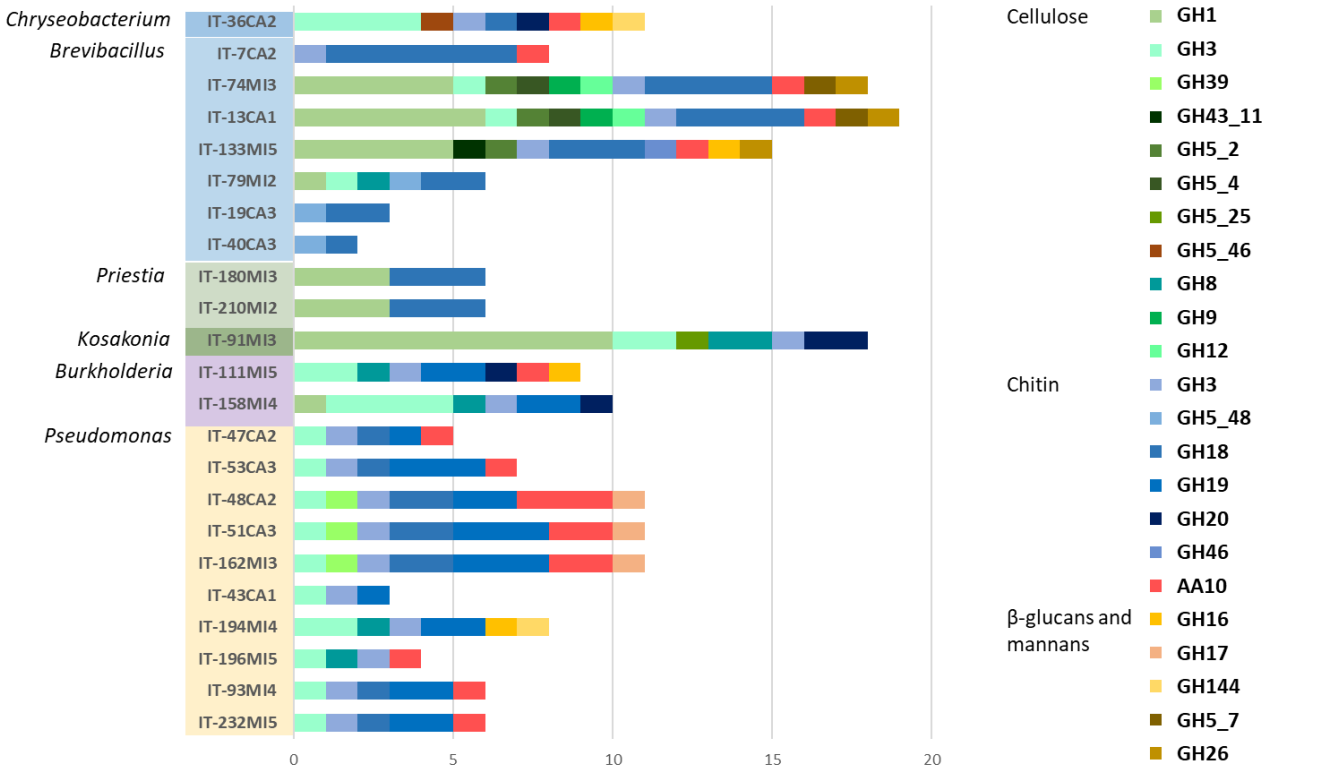

Figure S3. Abundance of genes belonging to CAZyme families potentially targeting cell wall components in fungi and oomycetes (cellulose, chitin, β-glucans and mannans) found in the genomes of bacterial isolates.

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CHAPTER 4

Fluorescent *Pseudomonas* from suppressive and non-suppressive soils share genomic and functional traits

AVANT-PROPOS

Soil represents the richest known reservoir of microbial biomass, and it is generally considered that less than 1% of the entire soil microbiome is culturable (Li et al., 2013). Among the soil-inhabiting phytobeneficial bacteria, fluorescent *Pseudomonas* species are one of the best studied and most promising in the context of biocontrol (Weller et al., 2007). *Pseudomonas* species are easy to isolate with standard microbiological techniques and have been extensively studied so far, with a constantly increasing number of newly described species. Whole genome sequencing technologies have contributed to developing tools such as Average Nucleotide Identity (ANI) and digital DNA-DNA hybridization (dDDH), that have become golden standards for species delineation and have contributed to description of many new *Pseudomonas* (and other) species (Lalucat et al., 2022). Fluorescent *Pseudomonas* are competitive root colonizers, well adapted to the rhizosphere environment (Haas & Défago, 2005), and are found in the plants rhizospheres in abundance, i.e., 10^6 CFU/g soil in bulk soil (Troxler et al., 1997a) and 10^{5-7} CFU/g root in the rhizosphere (Troxler et al., 1997b). They possess a large number of phytobeneficial functions (Haas & Défago, 2005; Loper et al., 2012), and their role is of particular importance in disease-suppressive soils, where it was shown that siderophore and phenazine-producing and systemic-resistance inducing *Pseudomonas* may contribute to suppressing *Fusarium* diseases (Scher & Baker, 1980; Mazurier et al., 2009; Lv et al., 2023). Besides investigating fluorescent *Pseudomonas* species in suppressive soils using traditional isolation techniques, metagenomic analysis of fluorescent pseudomonads communities may help reveal differences in suppressive vs. non-suppressive soils (Kyselková & Moënne-Loccoz, 2012), bearing in mind that phytobeneficial *Pseudomonas* may also be found in non-suppressive soils (Ramette et al., 2006; Frapolli et al., 2010).

In Chapter 3, we isolated and characterized bacteria of diverse taxonomy, from fungistatic and non-fungistatic soils, showing that non-fungistatic soils may also serve as a source of bacteria with biocontrol activity against *Fusarium graminearum*. However, in the present chapter, we had two objectives: (i) to describe novel *Pseudomonas* species, isolated from the wheat rhizospheres grown in Serbian soils, and (ii) to employ both culture-dependent and culture-independent methods as a tool in characterizing fluorescent *Pseudomonas* from suppressive vs. non-suppressive soils. To achieve our first objective, the whole-genome sequencing of *Pseudomonas* isolates, together with ANI and dDDH calculations were performed. Subsequently, genomes of novel species were annotated, and species were tested functionally. The novel species were deposited in three culture collections, i.e., Collection Française de Bactéries associées aux Plantes (CFBP), Belgian Coordinated Collections of Microorganisms (BCCM/LMG) and Environmental Microbiology Lyon - Biological Resource Centre (EML-BRC). These results were published in *Systematic and Applied Microbiology* in April, 2023. For our second objective, we used rhizospheres of non-inoculated wheat plants grown in suppressive and non-suppressive MI soils (MI2, MI3, MI4 and MI5; Chapter 2), we extracted the rhizospheric DNA and performed a metabarcoding analysis, targeting the *rpoD* gene of the *P. fluorescens* group, aiming to compare diversity and composition of the fluorescent group in suppressive vs. non-suppressive MI soils. Then, both *F. graminearum*-inoculated and non-inoculated rhizospheres of wheat grown in suppressive and non-suppressive MI soils (Chapter 2) were used to isolate 406 putative *Pseudomonas*, according to a protocol by Vacheron et al. (2016). We extracted the DNA from these putative *Pseudomonas* and characterized them based on *rpoD* gene, or *rrs* gene when *rpoD* gene amplification failed. Based on *rpoD* gene characterization, 29 *Pseudomonas* were chosen from all 8 soil conditions, i.e., 4 soils x inoculated/not with *F. graminearum*, and their genomes

were sequenced using the Illumina Nova Seq technology. After the sequencing, genomes of these bacteria were annotated, searching for genes known to be involved in biocontrol and plant growth promotion and carbohydrate-active enzymes (CAZymes) were predicted using dbCAN2 server. These chosen bacteria were also characterized functionally, by performing *in vitro* assays, including production of HCN and lytic enzymes, and production of ACC deaminase, phytohormones, siderophores and solubilization of phosphates. They were also assessed for their ability to inhibit *F. graminearum* conidia germination, as well as for their ability to produce VOCs that inhibit *F. graminearum* mycelial growth. Finally, putative biosynthetic gene clusters found in the 29 *Pseudomonas* genomes were identified using the antiSMASH and manually curated.

All of this work has led to the drafting of publication "Fluorescent *Pseudomonas* from suppressive and non-suppressive soils share genomic and functional traits" (submission scheduled for the end of 2023).

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PART A

Two novel species isolated from wheat rhizospheres in Serbia: *Pseudomonas serbica* sp. nov. and *Pseudomonas serboccidentalis* sp. nov.

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Two novel species isolated from wheat rhizospheres in Serbia: *Pseudomonas serbica* sp. nov. and *Pseudomonas serboccidentalis* sp. nov.

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ABSTRACT

Pseudomonas strains IT-194P, IT-215P, IT-P366^T and IT-P374^T were isolated from the rhizospheres of wheat grown in soils sampled from different fields (some of them known to be disease-suppressive) located near Mionica, Serbia. Phylogenetic analysis of the 16S rRNA genes and of whole genome sequences showed that these strains belong to two potentially new species, one containing strains IT-P366^T and IT-194P and clustering (whole genome analysis) next to *P. umsongensis* DSM16611^T, and another species containing strains IT-P374^T and IT-215P, and clustering next to *P. koreensis* LMG21318^T. Genome analysis confirmed the proposition of novel species, as ANI was below the threshold of 95% and dDDH below 70% for strains IT-P366^T (compared with *P. umsongensis* DSM16611^T) and IT-P374^T (compared with *P. koreensis* LMG21318^T). Unlike *P. umsongensis* DSM16611^T, strains of *P. serbica* can grow on D-mannitol, but not on pectin, D-galacturonic acid, L-galactonic acid lactone and α-hydroxybutyric acid. In contrary to *P. koreensis* LMG21318^T, strains of *P. serboccidentalis* can use sucrose, inosine and α-ketoglutaric acid (but not L-histidine) as carbon sources. Altogether, these results indicate the existence of two novel species for which we propose the names *Pseudomonas serbica* sp. nov., with the type strain IT-P366^T (= CFBP 9060^T = LMG 32732^T = EML 1791^T) and *Pseudomonas serboccidentalis* sp. nov., with the type strain IT-P374^T (= CFBP 9061^T = LMG 32734^T = EML 1792^T). Strains from this study presented a set of phytobeneficial functions modulating plant hormonal balance, plant nutrition and plant protection, suggesting a potential as *Plant Growth-Promoting Rhizobacteria* (PGPR).

Keywords: *Pseudomonas*, new species, Serbia

INTRODUCTION

The proteobacterial genus *Pseudomonas* consists of species with versatile metabolism and physiology, which are colonizing various aquatic, terrestrial and biotic environments. Since its discovery by Migula (1894), many new species have been added to this genus, now comprising more than 300 validly published species at the time of writing this manuscript (List of Prokaryotic Names with Standing in Nomenclature; https://lpsn.dsmz.de/genus/pseudomonas, accessed on 8 July 2022).

Representatives of the *Pseudomonas* genus display different lifestyles – some species are opportunistic human, insect or plant pathogens, some can be used in bioremediation, while others can act as PGPR by providing phytostimulation and/or phytoprotection functions (Silby et al., 2011). MultiLocus Sequence Analysis (MLSA) of four housekeeping genes (the 16S rRNA gene *rrs*, *gyrB*, *rpoB* and *rpoD*) and Average Nucleotide Identity (ANI) comparisons revealed three distinct lineages within the *Pseudomonas* genus – referred to as the *P. aeruginosa, P. fluorescens and P. pertucinogena* lineages (Peix et al., 2018). However, this classification contained inconsistencies, as the genus *Pseudomonas* was not monophyletic and included genera such as *Azotobacter* and *Azomonas* (Nikolaidis et al., 2020; Rudra and Gupta, 2021; Saati-Santamaría et al., 2021). In 2021, two articles published one month apart, developed a phylogenomic analysis of the genus *Pseudomonas*, proposing the reclassification of the monophyletic lineage of *P. pertucinogena*, which forms a clade distinct from the main *Pseudomonas* clade and consists of halotolerant species, into the genus '*Neopseudomonas*' (Saati-Santamaría et al., 2021) or *Halopseudomonas* (Rudra and Gupta, 2021). They also repositioned the deep branching species *Pseudomonas hussainii* into a new genus termed *'Parapseudomonas'* (Saati-Santamaría et al., 2021) or *Aptomonas* (Rudra and Gupta, 2021). Another phylogenomic study reclassified the *P. stutzeri* nitrogen-fixing clade and defined the new genus *Stutzerimonas* (Lalucat et al., 2022). Together with the *P. aeruginosa* lineage, the *P. fluorescens* lineage remains in the *Pseudomonas* genus. The *P. fluorescens* lineage (the most complex and diverse) is often subdivided into six phylogenetic groups, branching further into nine subgroups, represented by the species *P. fluorescens*, *P. gessardii*, *P. fragi*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata*, *P. chlororaphis* and *P. asplenii* (Mulet et al., 2010; Gomila et al., 2015; Peix et al., 2018). It is known that the *P. fluorescens* group contains a few phytopathogens (such as strains *P. corrugata* or *P. mediterranea*; Trantas et al., 2015) and various plant-beneficial species with properties such as siderophore production (Garrido-Sanz et al., 2016), phosphate solubilization (Meyer et al., 2010) and production of phytohormones (Vacheron et al., 2016; Keshavarz-Tohid et al., 2017), thus modulating plant growth. Besides, production of extracellular lytic enzymes such as chitinases, cellulases and proteases (Ali et al., 2020), as well as hydrogen cyanide (Frapolli et al., 2012) and various other antifungal secondary metabolites (Dutta et al., 2020) make some strains of the *P. fluorescens* group good candidates for biocontrol.

In the present study, four *Pseudomonas* strains were isolated from the rhizospheres of wheat grown in soils collected from different fields (some of them suppressive to *Fusarium graminearum* disease), located near Mionica, Serbia, and their genomes were fully sequenced in order to assign them at the species level. Genome sequence similarities of strains presented in this study and type strains from the closest, currently described *Pseudomonas* species were below the ANI and dDDH threshold levels established for differentiating bacterial species. These strains, belonging to the *P. fluorescens* group, were further described phenotypically and phylogenetically, and we propose the names *Pseudomonas serbica* (with IT-P366^T as type strain) and *Pseudomonas serboccidentalis* (with IT-P374^T as type strain) for these novel species.

MATERIAL AND METHODS

Isolation of bacterial strains and growth conditions

In the present work, four *Pseudomonas* strains were isolated from the rhizospheres of wheat grown in soils originating from farm fields near Mionica, Serbia, i.e., strain IT-194P from soil MI3, strain IT-215P from soil MI4, and strains IT-P366^T and IT-P374^T from a same plant in soil MI5 (Table S1), as follows. Wheat (*Triticum aestivum* L.) cv. Recital (provided by Thierry Langin, GDEC, INRAe, Clermont-Ferrand, France) was grown in these soils for 28 days in the greenhouse, wheat roots were harvested and shaken vigorously to discard loosely-adhering soil, and rhizosphere extracts were prepared using an adapted protocol from Bulgarelli et al. (2012). In brief, each wheat root system with adhering soil was put in 50 mL of phosphate buffered saline (NaCl, 8 g; KCl, 0.2 g; KH_2PO_4, 0.24 g; Na_2HPO_4, 1.42 g; H_2O, 1000 mL) and shaken for one hour at 160 rpm at room temperature. The roots were discarded and the suspension was centrifuged at 4000*xg* for 20 min. The resulting pellet was mixed with 20 mL of 0.8% NaCl, vortexed, and the suspension (i.e., rhizosphere extract) was serially diluted in liquid King's B^{+++} [i.e., King's B broth (Condalab, Madrid, Spain) supplemented with ampicillin (40 µg.mL^{-1}), chloramphenicol (13 µg.mL^{-1}) and cycloheximide (100 µg.mL^{-1}); Vacheron et al., 2016]. Following incubation for 24 h at 28°C, 1 µL from each of the last dilution (10^{-8}) displaying growth was plated on King's B agar (Condalab). Colonies were randomly picked and purified three times on King's B agar, yielding strains IT-194P, IT-215P, IT-P366^T and IT-P374^T (and others), which were preserved by deep-freezing in 25% glycerol. The four strains have been deposited in three culture collections, i.e., Collection Française de Bactéries associées aux Plantes (CFBP), Belgian Coordinated Collections of Microorganisms (BCCM/LMG) and Environmental Microbiology Lyon - Biological Resource Centre (EML-BRC, https://brclims.pasteur.fr/brcWeb/souche/recherche).

DNA extraction and genome sequencing

Genomic DNA extraction from all four strains was done from an overnight culture in Tryptone Soya Broth (TSB; Carl Roth, Karlsruhe, Germany) using the Nucleospin tissue kit (Macherey-Nagel, Hoerdt, France), according the manufacturer's instructions. Whole-genome sequences were determined by Novogene (Cambridge, England) using the Illumina NovaSeq technology, generating a 2 × 150 bp paired-end library. Fastp software (v.0.23.1; Chen et al., 2018) was used for trimming sequences (default settings) and Unicycler software (v.0.5.0; Wick et al., 2017) for *de novo* assembly. Genomic features of the strains were obtained using the MicroScope platform (Vallenet et al., 2020). Genome sequences of the type strains IT-P366^T and IT-P374^T were deposited in the GenBank database under the accession numbers PRJNA863439 and PRJNA859669, respectively. The 16S rRNA gene sequences *rrs* of the type strains IT-P366^T and IT-P374^T were extracted from the whole genome sequences and deposited in the GenBank database under the accession numbers OP021714 and OP021715, respectively.

Phylogenetic analyses

Type strain Genome Server (TYGS) (https://tygs.dsmz.de/; Meier-Kolthoff and Goker, 2019; Meier-Kolthoff et al., 2022) was used to construct phylogenetic trees based on whole-genome and *rrs* sequences of the four studied strains and other *Pseudomonas* strains present in the database. Briefly, the TYGS pipeline selects the closest type-strain genomes using two complementary ways. First, the four genomes that were assessed ('query') were compared with all available type-strain genomes in the TYGS database using the MASH algorithm, a fast approximation of intergenomic relatedness (Ondov et al., 2016), and the type strains with the lowest MASH distances per requested genome were selected. Second, the *rrs* sequences were used to identify an additional set of closely-related type strains. *rrs* sequences were extracted from the four genomes using RNAmmer (Lagesen and Hallin, 2007) and each *rrs* sequence was then Blasted (Camacho et al., 2009) against the 18799 type strains available in the TYGS database. This dual approach was used to find the 50 best matching type strains (based on the binary score) for each user genome, and then to calculate accurate distances using the Genome BLAST Distance Phylogeny (GBDP) method based on the coverage algorithm and the d5 distance formula (Meier-Kolthoff et al., 2013). These distances were then used to determine the 10 closest type-strain genomes for each query genome. The *rrs* phylogenetic tree was inferred with FastME 2.1.6.1 (Lefort et al., 2015) based on GBDP distances (Meier-Kolthoff et al., 2013). However, as only poor resolution of strains is often achieved with *rrs* sequences (Rodriguez-R et al., 2018), a phylogenetic tree with whole-genome sequences was also inferred, using FastME 2.1.6.1 (Lefort et al., 2015) and GBDP distances calculated from whole genome sequences. The trees were visualized using iTOL software (Letunic and Bork, 2016).

The genomic relatedness of the four strains used in this study to the type strains available in public databases was ascertained by calculating the Average Nucleotide Identity (ANI) and digital DNA–DNA Hybridization (dDDH) values. ANI by BLAST (ANIb) was calculated using the JSpecies server, based on BLAST (Richter et al., 2016) and dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0) (Meier-Kolthoff et al., 2013; Meier-Kolthoff et al., 2022), using the recommended BLAST method. The GGDC results were based on formula 2, which is independent of the genome length and is recommended to use for incomplete draft genomes. Recommended cut-off values for ANI and dDDH for prokaryotic species differentiation are 95-96% and 70%, respectively (Chun et al., 2018). Percentage of 16S rRNA gene identity of IT-P366^T and IT-P374^T with the closest type strains available in the public databases was calculated using the EzBioCloud server (Yoon et al., 2017). Additionally, pan-genome analysis was performed with the MicroScope platform (amino acid identity > 80%; alignment coverage > 80%) (Vallenet et al., 2020) to visualize the core and unique genes between strains of the same species presented in this study.

Morphological, biochemical and physiological tests

Gram staining was done with standard methods. Temperature range was determined by growing strains on Tryptone Soya Agar (TSA) (Condalab) at 4, 10, 37 and 41°C for 48 h. The range of pH suitable for growth was determined by inoculating Nutrient Broth (Condalab) with pH adjusted to 3, 4, 5, 6, 7, 8 and 9 and incubating for 48 h at 28°C. Oxidase activity was assessed using Oxidase test strips (Sigma Aldrich, St Louis, MO, USA) and catalase activity by resuspending one colony in a drop of 3% hydrogen peroxide and monitoring bubble production. Fluorescent pigment production was tested on King's B agar, *Pseudomonas* Agar F (PAF; BD Difco, Sparks, MD,

USA) and Potato Dextrose Agar (PDA; Condalab). Swimming, swarming and twitching motilities were checked by stab-inoculating media containing 0.3% meat extract, 0.5% peptone, 0.5% NaCl as well as 0.3%, 0.5% and 1.5% agar (pH 7), respectively, in triplicates. In brief, bacterial strains were grown overnight in TSB, 2 mL of cell suspension was transferred to 2 mL tube, centrifuged at 8000*xg* for 10 min, after which the supernatant was discarded. The pellet was stabbed with the tip of a sterile toothpick, inoculated in the middle of the plates containing different concentrations of agar and incubated for 48 h at 28°C. Further testing of these strains included phosphate solubilization on NBRIP media (National Botanical Research Institute's Phosphate), production of hydrogen cyanide (HCN; Bakker and Schippers, 1987) and production of extracellular protease on milk agar. Besides, characterization included assessment of siderophore production (protocol by Lakshmanan et al., 2015), production of cellulase (Guesmi et al., 2022) and ACC deaminase activity (according to Penrose and Glick, 2003). Screening for production of (i) seven auxin phytohormones, i.e., indole-3-acetic acid (IAA), indole-3-lactic acid, indole-3-carboxylic acid, indole-3-pyruvic acid, indol-3-butyric acid (IBA), tryptophol and indole-3-propionic acid, (ii) five cytokinins, i.e., trans-zeatin, trans-zeatin riboside (ZR), kinetin, 6-benzylaminopurine (BAP) and isopentenyl adenosine (IPA), (iii) two gibberellins, i.e., gibberellin A1 (GA1) and gibberellic acid (GA3), (iv) abscisic acid (ABA) and (v) kynurenic acid was done by Ultra High Performance Liquid Chromatography (UHPLC). Briefly, all isolates were grown 3 days at 28°C (300 rpm) in 2 mL of M9 minimal medium (Elbing and Brent, 2002) supplemented with 0.4 mM of tryptophan and 0.1 mM of adenine. The cultures were centrifuged at 4500*xg* during 8 min and filtered at 0.2 µm. Supernatants were lyophilized (Alpha 1–4 LSC Martin Christ, Osterode, Germany) for 24 h, the powder obtained was extracted two times with methanol, drying with speed-vac (Centrivap Cold Trap Concentrator LABCONCO, Kansas City, MO, USA), and UHPLC separation was performed with an Agilent 1290 Series instrument (Agilent Technologies France, Les Ulis, France) using a 100 × 3 mm reverse phase column (Agilent Poroshell 120 EC-C18, 2.7 µm particle size). Samples (3 µL) were loaded onto the column equilibrated with solvent A (water + 0.4% formic acid) and solvent B (acetonitrile) in a 98:2 ratio. Compounds were eluted by increasing the acetonitrile concentration to 40% over a 6 min period, then to 100% over 4 min, followed by an isocratic step of 2 min, at a flow rate of 0.5 $mL.min^{-1}$. Hormones were detected with a diode array detector (DAD) and an Agilent 6530 Q-TOF mass spectrometer in positive and negative electrospray ionization, based on comparison with commercial standards on both mass and UV (between 190 and 600 nm) chromatograms, along with accurate mass and UV spectra. Further phenotypic tests were performed with Biolog GEN III MicroPlates (Biolog, Hayward, CA, USA), API 20 NE and API ZYM strips (BioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions.

Antibiotic susceptibility

Strains in this study were tested for their susceptibility to 10 different antibiotics using the disc diffusion method (Bauer, 1966). The antibiotic discs (Bio-Rad, Marnes-la-Coquette, France) included imipenem (10 µg), cefepime (30 µg), amikacin (30 µg), ticarcillin (75 µg), ticarcillin/clavulanic acid (75 + 10 µg), tobramycin (10 µg), meropenem (10 µg), aztreonam (30 µg), ciprofloxacin (5 µg) and levofloxacin (5 µg). In brief, the antibiogram was done by plating bacterial suspension (density at 625 nm adjusted to 0.5 McFarland units with sterile saline solution) on Mueller Hinton medium (MH; Condalab) and firmly applying antibiotic disks on the agar surface. Inverted plates with antibiotic discs were incubated for 24 h at 33°C (as recommended by EUCAST and SFM, Manual v.1.0. May 2022, CASFM2022_V1.0.pdf; sfm-

microbiologie.org). After incubation, antibiotic sensitivity or resistance was evaluated by measuring zones of inhibition and comparing to critical values for *Pseudomonas aeruginosa* available at European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org).

Genome analyses

Genome annotation was done automatically with the MicroScope platform (v.3.15.4; Vallenet et al., 2020). DIAMOND blastp (v.2.0.8.146; Buchfink et al., 2015) was used to search for genes involved in biocontrol and plant-growth promotion (accession numbers are available in Chapter 3; Table S1) within genome protein sequences using the options --query-cover 80 --id 70, in order to filter the hits with minimum 80% query coverage and minimum 70% amino acid identity. The searched functions and the corresponding genes were as follows: (i) production of antimicrobial compounds phenazine (*phzABCDEFG*) (Dar et al., 2020), 2-hexyl-5-propyl-alkylresorcinol (*darABC*) (Nowak-Thompson et al., 2003), 2,4-diacetylphloroglucinol (*phlABCD*) (Bangera and Thomashow, 1999), pyrrolnitrin (*prnABCD*) (Kirner et al., 1998), HCN (*hcnABC*) (Ramette et al., 2003) and pyoluteorin (*pltABCDEFGLM*) (Nowak-Thompson et al., 1999), (ii) production of insect toxin FitD (*fitD*) (Loper et al., 2012) and alkaline metalloproteinase AprA (*aprA*) (Loper et al., 2012) involved in biocontrol, (iii) production of siderophores pyoverdine (*pvdL*) (Schalk and Guillon, 2013), pyochelin (*pchABCDEF*) (Reimmann et al., 2001) and pseudomonine (*pmsABCE*) (Matthijs et al., 2009), (iv) signaling and modulation of plant hormonal balance by deamination of ethylene precursor 1-aminocyclopropane-1-carboxylate (*acdS*) (Loper et al., 2012), ethylene production (*efe*) (Wang et al., 2010), auxin biosynthesis (*iaaMH*, *ipdC*/*ppdC*) (Loper et al., 2012; Gruet et al., 2022), auxin catabolism (*iacABCDEFGHI*) (Loper et al., 2012), acetoin synthesis (*budB*/*ilvNB*/*alsS*, *budA*/*alsD*) (Loper et al., 2012; Blomqvist et al., 1993), 2,3-butanediol synthesis (*budC*/*ydjL* in addition to the acetoin synthesis genes) (Nicholson, 2008), 2,3-butanediol conversion to acetoin (*adh*/*bdhA*/*ydjL*) (Huang et al., 1994; Nicholson, 2008), acetoin catabolism (*acoABCX*) (Huang et al., 1994), (v) transformation of P and N sources by phosphate solubilization (*gcd*, *gad*) (Miller et al., 2010), nitrogen fixation (*nifHDK*) (Bruto et al., 2014) and denitrification (*nirK*, *nirS*) (Bruto et al., 2014; Coyne et al., 1989). In case where presence of more than one gene is necessary to achieve a function (*e.g.,* presence of both *iaaM* and *iaaH* for the synthesis of auxin via indole-3-acetamide pathway) and only some of the necessary genes were found in the genome, we checked for the presence of the missing genes with less stringent blast result filtering criteria (--query-cover 80 --id 30). Putative biosynthetic gene clusters were further identified using antiSMASH (Blin et al., 2019) within the MicroScope platform and the annotations were manually curated.

RESULTS AND DISCUSSION

Morphological, biochemical and physiological features

The cells from *P. serbica* and *P. serboccidentalis* species are Gram-negative. Their colonies are circular, beige, 2–3 mm in diameter after 48 h of incubation at 28°C on TSA medium. They are catalase and oxidase positive, and do not produce fluorescent pigment on King's B or PAF medium, but they produce it on PDA. All the strains show growth at 4°C, 10°C and 37°C, but not at 41°C, and they grow at pH 5 to 9, with an optimum at pH 7. All strains are strictly aerobic. All strains are motile by swimming movements, on plate with 0.3% agar. Results for API 20 NE and API ZYM strips are given in Table 1 and those for Biolog GEN III in Table 2 and Table S2, in

comparison with literature data for *P. koreensis* LMG21318^T (Morimoto et al., 2020) and *P. umsongensis* DSM16611^T (Furmanczyk et al., 2018). Strains of *P. serbica* species have the ability to grow using D-mannitol, but cannot grow on pectin, D-galacturonic acid, L-galactonic acid lactone or α-hydroxybutyric acid, and in the presence of 8% NaCl, in contrary to *P. umsongensis* DSM16611^T. Strains of *P. serboccidentalis* species have the ability to grow using sucrose, inosine or α-ketoglutaric acid, but cannot use L-histidine as a source of carbon, contrarily to *P. koreensis* LMG21318^T. All strains share features that are typical for *Pseudomonas* (Furmanczyk et al., 2018), such as the use of simple sugars (fructose and glucose), amino acids (L-alanine, L-arginine, L-aspartic acid and L-glutamic acid) and carboxylic acids (such as L-lactic acid, citric acid, L-malic acid and acetic acid) as sources of carbon. However, none of the four strains studied are able to use di-, tri- or tetrasaccharides (such as D-cellobiose, D-turanose, stachyose, D-raffinose, α-D-lactose), or D-salicin, *N*-acetyl-D-mannosamine, *N*-acetyl-neuraminic acid, D-sorbitol, D-glucose-6-phosphate, D-aspartic acid, D-lactic acid methyl ester, α-ketobutyric acid and acetoacetic acid.

Strains of *P. serbica* and *P. serboccidentalis* species are resistant to ticarcilline, ticarcilline/clavulanic acid and aztreonam (Table S3). However, for aztreonam a difference in resistance level was observed between *P. serbica* IT-P366T and IT-194P, strain IT-194P being fully resistant to aztreonam.

Table 1. Phenotypic characteristics of *Pseudomonas serbica* and *Pseudomonas serboccidentalis*. For each species, data were obtained from the type strain and one related strain.

	P. serbica	*P. serboccidentalis*
General properties		
Fluorescence on PDA	+	+
Fluorescence on King's B agar	-	-
Fluorescence on PAF agar	-	-
Oxidase	+	+
Catalase	+	+
Enzyme activities (API ZYM)		
Alkaline phosphatase	+	-
Esterase (C 4)	+	+
Esterase Lipase (C 8)	+	+
Lipase (C 14)	-	-
Leucine arylamidase	+	+
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α-Chymotrypsin	-	-
Acid phosphatase	+	+
Naphthol-AS-BI-phosphohydrolase	+	+
α-Galactosidase	-	-
β-Galactosidase	-	-
β-Glucuronidase	-	-
α-Glucosidase	-	-
β-Glucosidase	-	-
N-Acetyl-β-glucosaminidase	-	-
α-Mannosidase	-	-
α-Fucosidase	-	-

Metabolism (API 20 NE)		
Nitrate reduction	+	-
Indole production from L-tryptophane	-	-
D-Glucose fermentation	-	-
L-Arginine dihydrolase	-	-
Urease	-	-
Esculin ferric citrate hydrolysis	-	
Gelatin hydrolysis	-	+
β-galactosidase	-	-
Growth on C sources (API 20NE)	-	-
D-Glucose assimilation	+	+
L-Arabinose assimilation	+	+
D-Mannose assimilation	+	+
D-Mannitol assimilation	+	+
N-Acetyl-glucosamine assimilation	d	+
D-Maltose assimilation	d	-
Potassium gluconate assimilation	+	+
Capric acid assimilation	+	+
Adipic acid assimilation	-	-
Malic acid assimilation	+	+
Trisodium citrate assimilation	+	+
Phenylacetic acid assimilation	+	-
Production of phytohormones		
Indole-3-acetic acid (IAA)	-	+
Indole-3-lactic acid	-	+
Indole-3-carboxylic acid	-	+
Indole-3-pyruvic acid	-	-
Indole-3-butyric acid (IBA)	-	-
Tryptophol	-	-
Indole-3-propionic acid	-	-
Trans-zeatin	+	+
Trans-zeatin riboside (ZR)	d	d
Kinetin	-	-
6-Benzylaminopurine (BAP)	+	d
Isopentenyl adenosine (IPA)	+	d
Gibberellin A1 (GA1)	-	-
Gibberellic acid (GA3)	-	-
Abscisic acid (ABA)	+	-
Kynurenic acid	+	d
Other tests		
Solubilization of phosphates	+	d
HCN production	-	+
Production of siderophores	+	+
Production of extracellular protease	-	d
Production of cellulase	-	-
Production of ACC deaminase	-	-

-, negative; +, positive; d, depends on the tested strain

Table 2. Selected differential phenotypic characteristics of *Pseudomonas serbica* and *Pseudomonas serboccidentalis*, determined by Biolog GEN III microplates. For each species, data were obtained from the type strain and one related strain. Literature data are shown for *Pseudomonas umsongensis* DSM16611^T (Furmanczyk et al., 2018) and *Pseudomonas koreensis* LMG21318^T (Morimoto et al., 2020). A complete list of phenotypic characteristics is presented in Table S2.

Biolog GEN III	*P. umsongensis* DSM16611^T	*P. serbica*	*P. koreensis* LMG21318^T	*P. serboccidentalis*
Carbon sources				
Sucrose	-	d	-	+
D-Fucose	-	d	-	w
Inosine	-	d	-	+
D-Mannitol	-	+	+	w
D-Serine	-	d	-	w
L-Histidine	+	+	+	-
Pectin	+	-	-	-
D-Galacturonic acid	+	-	-	-
L-Galactonic acid lactone	+	-	-	-
Glucuronamide	+	w	-	w
α-Ketoglutaric Acid	+	+	-	+
Tween 40	+	w	-	w
α-Hydroxybutyric acid	+	-	-	-
Other Biolog GEN III tests				
8% NaCl	+	-	-	w
Minocycline	-	d	-	+
Sodium bromate	+	d	-	w

-, negative; +, positive; d, depends on the tested strain; w, weak

Phylogenetic and genomic analyses

The phylogenetic tree inferred from *rrs* sequences (Figure 1) showed that IT-P366^T and IT-194P clustered together with *P. mohnii*, and IT-P374^T and IT-215P formed a cluster close to *P. gozinkensis*, *P. granadensis*, *P. monsensis*, *P. allokribbensis*, *P. glycinae*, *P. fitomaticsae* and *P. kribbensis*.

Figure 1. Phylogenetic tree of housekeeping gene *rrs* gene showing the relation of *Pseudomonas serbica* strains IT-P366^T (in bold) and IT-194P and *Pseudomonas serboccidentalis* strains IT-P374^T (in bold) and IT-215P with representative strains of *Pseudomonas*. The tree was constructed using TYGS server, inferred with FastME 2.1.6.1 (Lefort et al., 2015) from Genome BLAST Distance Phylogeny (GBDP) distances, calculated from *rrs* gene sequences. Numbers at the branching points are GBDP pseudo-bootstrap support values > 60% from 100 replications. The tree was visualized using iTOL software (Letunic and Bork, 2016). *Cellvibrio japonicus* Ueda 107^T was used as the outgroup. Accession numbers for all of the type strains used to construct the tree are given in Table S4.

However, when the phylogenetic tree was inferred from whole-genome sequences (using TYGS) (Figure 2), the closest species to strains IT-P366^T and IT-194P was in fact *P. umsongensis*, and the closest species to strains IT-P374^T and IT-215P was *P. koreensis*. The same results were obtained with a phylogeny based on MLSA (Figure S1). Accession numbers for all of the strains used to construct the tree are given in Table S4.

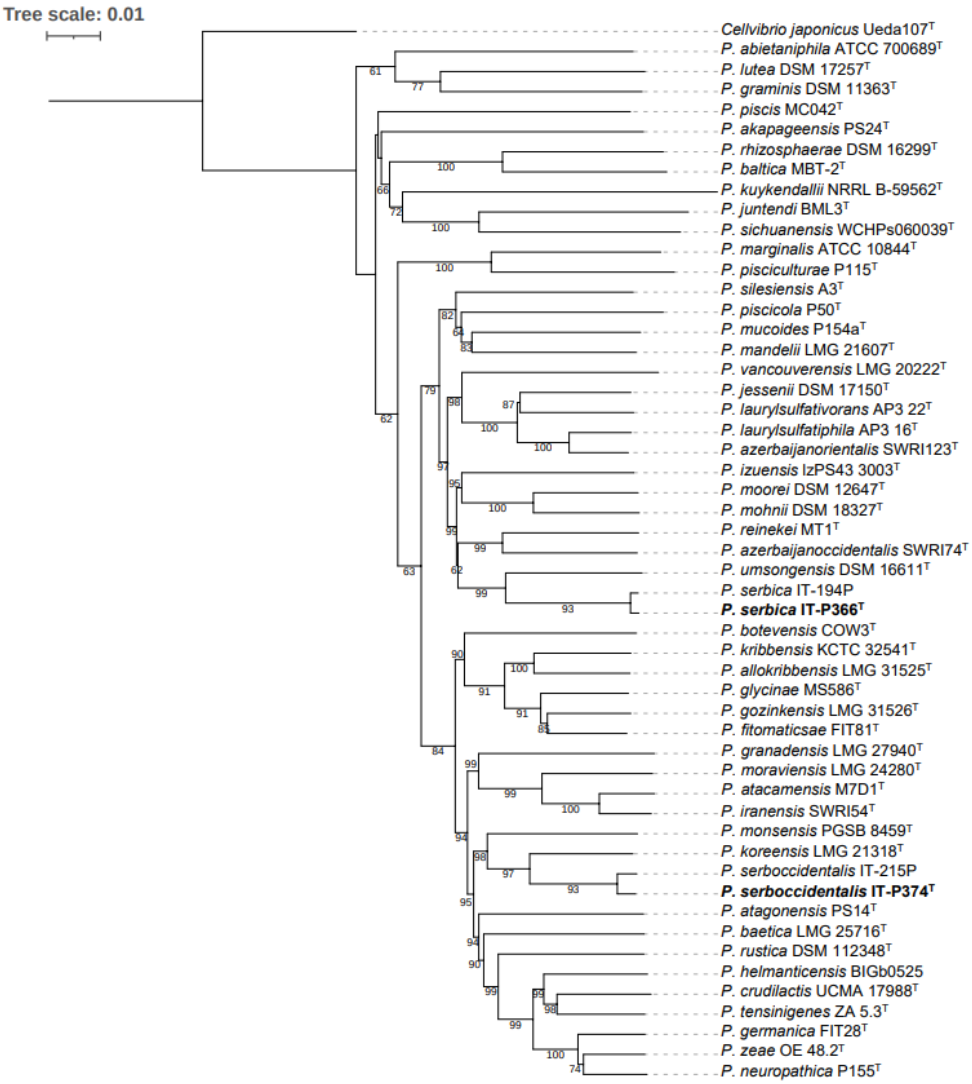

Figure 2. Phylogenetic tree using whole-genome sequences showing the relation of *Pseudomonas serbica* strains IT-P366^T (in bold) and IT-194P and *Pseudomonas serboccidentalis* strains IT-P374^T (in bold) and IT-215P with representative strains of *Pseudomonas*. The tree was constructed using TYGS server, inferred with FastME 2.1.6.1 (Lefort et al., 2015) from GBDP distances, calculated from genome sequences. Numbers at the branching points are GBDP pseudo-bootstrap support values > 60% from 100 replications. The tree was visualized using iTOL software (Letunic and Bork, 2016). *Cellvibrio japonicus* Ueda 107^T was used as the outgroup. Accession numbers for all of the type strains used to construct the tree are given in Table S4.

The proposition of two new species was based on dDDH values (computed with GGDC 3.0 and formula 2) for strains IT-P366^T (proposed type strain for *P. serbica*) and IT-P374^T (proposed type strain for *P. serboccidentalis*), which were lower than the threshold of 70% when comparing with the closest type strains available in the database (Table 3 and Table 4).

Table 3. Average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values of *Pseudomonas serbica* IT-P366^T and IT-194P with the closest type strains (as seen in Figure 2). dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0) (Meier-Kolthoff et al., 2013; Meier-Kolthoff et al., 2022), using the recommended BLAST method. For ANIb calculations, genomes from *Pseudomonas umsongensis* DSM 16611^T, *Pseudomonas azerbaijanoccidentalis* SWRI74^T, *Pseudomonas reinekei* MT1^T, *Pseudomonas mohnii* DSM 18327^T, *Pseudomonas moorei* DSM 12647^T and *Pseudomonas izuensis* lzPS43_3003^T were available at the JSpecies server (Richter et al., 2016). Calculation of % of 16S rRNA identity of IT-P366^T and the closest type strains was done using the EzBioCloud server (Yoon et al., 2017).

	% 16S rRNA identity with type strain IT-P366^T	IT-P366^T		IT-194P	
		ANI	dDDH	ANI	dDDH
P. umsongensis DSM 16611^T	99.63	89.52	41	89.61	41.10
P. azerbaijanoccidentalis SWRI74^T	98.31	86.14	33.30	86.05	33.30
P. reinekei MT1^T	98.36	86.37	33.50	86.38	33.50
P. mohnii DSM 18327^T	100	85.93	33	85.93	33.10
P. moorei DSM 12647^T	99.81	85.92	33	85.94	33
P. izuensis lzPS43_3003^T	99	85.53[a]	33.20	85.63[a]	33.20
P. serbica IT-P366^T				98.70	95.10
P. serbica IT-194P	100	98.74	95.10		

[a] Genome coverage for ANIb calculations between each comparison was > 69%, except in the case of *P. izuensis* lzPS43_3003 and *P. serbica* strains IT-P366^T and IT-194P, where the genome coverage between *P. izuensis* lzPS43_3003 and these strains was 67.95 and 68.02, respectively.

Table 4. Average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values of *Pseudomonas serboccidentalis* IT-P374^T and IT-215P with the closest type strains (as seen in Figure 2). dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (GGDC 3.0) (Meier-Kolthoff et al., 2013; Meier-Kolthoff et al., 2022), using the recommended BLAST method. For ANIb calculations, genomes from *Pseudomonas koreensis* LMG21318^T and *Pseudomonas monsensis* PGSB 8459^T were available at the JSpecies server (Richter et al., 2016), and the genome coverage between each comparison was > 69%. Calculation of % of 16S rRNA identity of IT-P374^T and the closest type strains was done using the EzBioCloud server (Yoon et al., 2017).

	% 16S rRNA identity with type strain IT-P374^T	IT-P374^T		IT-215P	
		ANI	dDDH	ANI	dDDH
Pseudomonas koreensis LMG 21318^T	99.79	91.86	48.60	91.88	48.50
Pseudomonas monsensis PGSB 8459^T	99.34	88.58	38.70	88.48	38.40
P. serboccidentalis IT-P374^T				98.47	88.10
P. serboccidentalis IT-215P	100	98.45	88.10		

Furthermore, ANIb values with the closest related strains were 89.52% for strain IT-P366^T and 91.86% for strain IT-P374^T, which is below the species-delimiting threshold of 95-96%. These criteria were also passed by the strains IT-194P (proposed *P. serbica*, Table 3) and IT-215P (proposed *P. serboccidentalis*, Table 4). In contrary, dDDH and ANIb values were 95.10 and 98.74 for strains IT-P366^T and IT-194P, respectively (within the proposed *P. serbica*), and 88.10 and 98.45 for IT-P374^T and IT-215P, respectively (within the proposed *P. serboccidentalis*), thus confirming that these pairs of strains belonged to the same two species.

The main genomic features of *P. serbica* IT-P366^T and *P. serboccidentalis* IT-P374^T are a genome size of respectively 7602 and 5997 kb, with respectively 7592 and 5580 protein-coding genes, and a GC content of respectively 59.5% and 60.4% (Table 5). The genome size of *P. serbica* IT-P366^T is almost 1 Mbp above that of the closest type strain *P. umsongensis* DSM 16611^T. A megaplasmid of 1,059,298 bp identified in strain IT-P366^T is absent from the genome of the second strain IT-194P of the proposed species *P. serbica*. The presence of this plasmid partly explains the large size difference between the genomes of the two strains (792,935 bp). Megaplasmids are rare in *Pseudomonas*, but they can allow the host cell to expand its specific niche (Kuepper et al., 2015; Purtschert-Montenegro et al., 2022). Thus, this megaplasmid hosts an operon for the synthesis of a type IVB secretion system (Dot/Icm family; Costa et al., 2021), whose homolog was recently described to be involved in the biocontrol of a bacterial pathogen (Purtschert-Montenegro et al., 2022), a chemotaxis operon and a flagellum synthesis operon. These functions represent an addition to the core species functions encoded in the chromosome of *P. serbica*, which contains another flagellum synthesis operon (identical to the one of IT-194P). In contrast, the genome sizes of *P. serboccidentalis* IT-P374^T and IT-215P are similar. The GC content is comparable in all the strains (Table 5).

Table 5. Genomic characteristics of *Pseudomonas serbica* IT-P366^T and *Pseudomonas serboccidentalis* IT-P374^T and their closest type strains. Genomic features of strains IT-P366^T and IT-P374^T were obtained from the MicroScope platform (Vallenet et al., 2020) and those from species *Pseudomonas koreensis* LMG21318^T, *Pseudomonas monsensis* PGSB 8459^T, *Pseudomonas umsongensis* DSM 16611^T, *Pseudomonas azerbaijanoccidentalis* SWRI74^T, *Pseudomonas reinekei* MT1^T, *Pseudomonas mohnii* DSM 18327^T, *Pseudomonas moorei* DSM 12647^T and *Pseudomonas izuensis* lzPS43_3003^T from the GenBank database.

Strains	GeneBank BioProject ID	Genome size (bp)	No. contigs	Plasmid (bp)	GC-content (%)	Protein-coding genes (CDS)
IT-P366^T	PRJNA863439	7,601,897	93	1,059,298	59.5	7592
IT-P374^T	PRJNA859669	5,997,322	39	0	60.4	5580
P. koreensis LMG 21318^T	PRJDB10510	6,064,848	41	0	60.5	5435
P. monsensis PGSB 8459^T	PRJNA639797	6,422,728	2	0	60	5533
P. umsongensis DSM 16611^T	PRJNA390488	6,701,403	14	0	59.7	5865
P. azerbaijanoccidentalis SWRI74^T	PRJNA639797	6,742,611	29	0	59.3	6015
P. reinekei MT1^T	PRJNA359931	6,249,573	63	0	59.1	5566
P. mohnii DSM 18327^T	PRJEB16418	6,592,588	2	0	59.6	5882
P. moorei DSM 12647^T	PRJNA563568	6,546,438	59	0	59.6	5877
P. izuensis lzPS43_3003^T	PRJNA594796	6,857,708	129	0	59.6	6093

Pan-genome analysis indicated that strains IT-P366^T and IT-194P shared 5553 genes, and strains IT-P374^T and IT-215P shared 5115 genes. Besides, the numbers of unique genes per strain were as follows: 1913 in IT-P366^T, 946 in IT-194P, 408 in IT-P374^T, and 602 in IT-215P.

Genome analyses revealed the presence of several genes and gene clusters related to phytobeneficial functions in the strains of *P. serbica* and *P. serboccidentalis* (Table 6). Both strains of *P. serbica* possessed genes involved in the modulation of plant hormonal levels, notably *iaaMH* genes for auxin biosynthesis and the gene *efe* for ethylene production. Both strains of *P. serboccidentalis* had a gene cluster for HCN production and the *aprA* gene for alkaline metalloproteinase involved in biocontrol. The two strains also possessed glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gad*) genes for the production of gluconic and 2-ketogluconic acids involved in phosphate solubilization. In addition, strains IT-194P (*P. serbica*), IT-215P and IT-P374^T (*P. serboccidentalis*) harbored the conserved gene *pvdL* for production of siderophore pyoverdine.

Table 6. Distribution in *Pseudomonas serbica* IT-P366^T and IT-194P and *Pseudomonas serboccidentalis* IT-P374^T and IT-215P of genes involved in biocontrol and plant-growth promotion. Presence of the property was marked with the gene name, found with DIAMOND blastp (v.2.0.8.146; Buchfink et al., 2015), using the options --query-cover 80 --id 70 (query coverage >80%; amino acid identity >70%), if not specified otherwise.

Strains	HCN production	Alkaline metalloproteinase production	Phosphate solubilization	Pyoverdine production	Ethylene production	Auxin biosynthesis
P. serbica						
IT-P366^T					*efe*	*iaaMH**
IT-194P				*pvdL*	*efe*	*iaaMH**
P. serboccidentalis						
IT-P374^T	*hcnABC*	*aprA*	*gcd, gad*	*pvdL*		
IT-215P	*hcnABC*	*aprA*	*gcd, gad*	*pvdL*		

Genes (and functions) that we searched for, but were not found in any of the described strains, were as follows: *phzABCDEFG* (production of phenazine), *darABC* (production of 2-hexyl-5-propyl-alkylresorcinol), *phlABCD* (production of 2,4-diacetylphloroglucinol), *prnABCD* (production of pyrrolnitrin), *pltABCDEFGLM* (production of pyoluteorin), *fitD* (production of insect toxin), *pchABCDEF* (production of pyochelin), *pmsABCE* (production of pseudomonine), *acdS* (production of aminocyclopropane-1-carboxylate deaminase), *ipdC* and *ppdC* (auxin biosynthesis), *iacABCDEFGHI* (auxin catabolism), *budB*/*ilvNB*/*alsS* and *budA*/*alsD* (acetoin synthesis), *budC*/*ydjL* (2,3-butanediol synthesis), *adh*/*bdhA*/*ydjL* (2,3-butanediol conversion to acetoin), *acoABCX* (acetoin catabolism), *nifHDK* (nitrogen fixation) and *nirK*, *nirS* (denitrification).
**iaaH* found only with 33% identity to the query.

Further search for secondary metabolite biosynthesis clusters by antiSMASH revealed that strain IT-P366^T also harbored *pvdL* gene involved in pyoverdine production. In addition, antiSMASH showed that the proposed species *P. serbica* (strains IT-P366^T and IT-194P) harbor species-specific gene clusters involved in testosterone degradation, previously described in *Comamonas testosteroni* Y1, isolated from activated sludge (Li et al., 2022) and in several manure-borne proteobacterial species, but not in *Pseudomonas* (Yang et al., 2011). Besides, strains P366^T and IT-194P have genes *bcsABGQZ*, known to be involved in the synthesis of cellulose, which is contributing to biofilm formation and promoting the epiphytic lifestyle of *P. syringae* (Arrebola et al., 2015).

Preliminary dual-confrontation plate assays indicated that *P. serbica* IT-P366^T and IT-194P and *P. serboccidentalis* IT-P374^T and IT-215P did not inhibit growth of the wheat phytopathogen *Fusarium graminearum* Fg1 on PDA (unpublished), suggesting that phytoprotection effects (if any) would probably entail indirect mechanisms mediated via plant metabolism. In addition, *P. serbica* and *P. serboccidentalis* possess genes for synthesis of mangotoxin, a virulence factor in *Pseudomonas syringae* (Passera et al., 2019), but in our hands these strains did not have any deleterious effect on wheat.

CONCLUSION

Based on the phylogenetic, genomic and phenotypic characteristics presented in this study, we showed the existence of two novel species within the genus *Pseudomonas*, for which the names *P. serbica* (with the type strain IT-P366^T) and *P. serboccidentalis* (with the type strain IT-P374^T) are proposed. The full protologue descriptions of these novel species are presented in Tables 7 and 8.

Table 7. Protologue description of *Pseudomonas serbica* sp. nov.

Genus name	*Pseudomonas*
Species name	*Pseudomonas serbica*
Specific epithet	*serbica*
Species status	sp. nov.
Species etymology	ser'bi.ca. N.L. fem. adj. serbica, pertaining to Serbia
Nature of the type material	strain
Description of the new taxon and diagnostic traits	Gram-negative rods, non-spore-forming and motile, oxidase and catalase positive. Colonies are circular, beige coloured, with 2–3 mm in diameter after 48h of incubation at 28°C on TSA medium. Temperature range for growth is 4°C to 37°C with optimum growth at 28°C. Strictly aerobic. The pH range for growth is 5 to 9 with optimum growth at pH 7.0. Positive tests with Biolog GEN III: pH 5, pH 6, 1% NaCl, 4% NaCl, α-D-Glucose, D-Mannose, D-Fructose, 1% Sodium Lactate, Fusidic Acid, D-Serine, D-Mannitol, Glycerol, Troleandomycin, Rifamycin SV, L-Alanine, L-Arginine, L-Aspartic Acid, L-Glutamic Acid, L-Histidine, L-Pyroglutamic Acid, L-Serine, Lincomycin, Guanidine hydrochloride, Niaproof 4, D-Gluconic Acid, Mucic Acid, Quinic Acid, D-Saccharic Acid, Vancomycin, Tetrazolium Violet, Tetrazolium Blue, L-Lactic Acid, Citric Acid, α-Ketoglutaric Acid, L-Malic acid, Potassium Tellurite, γ-Amino-N-Butyric Acid, β-Hydroxybutyric Acid, Acetic Acid and Aztreonam. Weak tests with Biolog GEN III: Glucuronamide, Methyl Pyruvate, Bromosuccinic Acid, Tween 40 and Formic acid. Negative tests with Biolog GEN III: D-Cellobiose, Gentiobiose, D-Turanose, Stachyose, D-Raffinose, α-D-Lactose, D-Melibiose, β-Methyl-D-Glucoside, D-Salicin, *N*-Acetyl-D-Mannosamine, *N*-Acetyl-Neuraminic Acid, 8% NaCl, 3-Methyl glucose, L-Fucose, L-Rhamnose, D-Sorbitol, D-Arabitol, D-Glucose-6-Phosphate, D-Fructose-6-Phosphate, D-Aspartic Acid, Pectin, D-Galacturonic Acid, L-

	Galactonic Acid Lactone, D-Lactic Acid Methyl Ester, α-Hydroxybutyric Acid, α-Ketobutyric Acid and Acetoacetic Acid. Variable tests with Biolog GEN III: Dextrin, Maltose, D-Trehalose, Sucrose, *N*-Acetyl-D-Glucosamine, *N*-Acetyl-D-Galactosamine, D-Galactose, D-Fucose, Inosine, myo-Inositol, D-Serine, Minocycline, Gelatin, Glycyl-L-Proline, D-Glucuronic Acid, *p*-Hydroxyphenyl Acetic Acid, D-Malic acid, Nalidixic Acid, Lithium Chloride, Propionic Acid, Sodium Butyrate and Sodium Bromate.
Country of origin	Serbia
Region of origin	Mionica, Western Serbia
Date of isolation	26/10/2021
Source of isolation	Rhizosphere of wheat (*Triticum aestivum* L.)
Sampling date	9/06/2021
Latitude	44.24759 N
Longitude	20.09931 E
Altitude	189 m
16S rRNA gene accession nr.	OP021714
Genome accession number	GenBank accession number: PRJNA863439
Genome status	Incomplete
Genome size	7,601,897 bp
GC mol%	59.5%
Number of strains in study	02
Source of isolation of non-type strains	Rhizosphere of wheat (*Triticum aestivum* L.)
Information related to the Nagoya Protocol	Implementation of Nagoya Protocol is still not fully in place in Serbia, Mr. Dusan Ognjanovic (Serbian representative for agreements on biological and genetic resources) was contacted regarding this issue.
Designation of the Type Strain	IT-P366^T
Strain Collection Numbers	CFBP 9060^T, LMG 32732^T, EML 1791^T

Table 8. Protologue description of *Pseudomonas serboccidentalis* sp. nov.

Genus name	*Pseudomonas*
Species name	*Pseudomonas serboccidentalis*
Specific epithet	*serboccidentalis*
Species status	sp. nov.
Species etymology	serb.oc.ci.den.ta'lis. N.L. fem. n. Serbia, a Balkan country; L. masc. adj. occidentalis, western; N.L. fem. adj. serboccidentalis, pertaining to western Serbia
Nature of the type material	strain
Description of the new taxon and diagnostic traits	Gram-negative rods, non-spore-forming and motile, oxidase and catalase positive. Colonies are circular, beige coloured, with 2–3 mm in diameter after 48h of incubation at 28 °C on TSA medium. Temperature range for growth is 4 °C to 37 °C with optimum growth at 28 °C. Strictly aerobic. The pH range for growth is 5 to 9 with optimum growth at pH 7.0. Positive tests with Biolog GEN III: Sucrose, pH 5, pH 6, 1% NaCl, 4% NaCl, α-D-Glucose, D-Mannose, D-Galactose, Inosine, 1% Sodium Lactate, Fusidic Acid, D-Serine, Troleandomycin, Rifamycin SV, Minocycline, L-Alanine, L-Arginine, L-Aspartic Acid, L-Glutamic Acid, L-Pyroglutamic Acid, Lincomycin, Guanidine hydrochloride, Niaproof 4, D-Gluconic Acid, Mucic Acid, Quinic Acid, D-Saccharic Acid, Vancomycin, Tetrazolium Violet, Tetrazolium Blue, L-Lactic Acid, Citric Acid, α-Ketoglutaric Acid, L-Malic Acid, Nalidixic Acid, Lithium Chloride, Potassium Tellurite, γ-

	Amino-N-Butyric Acid, β-Hydroxybutyric Acid, Propionic Acid, Acetic Acid and Aztreonam. Weak tests with Biolog GEN III: *N*-Acetyl-D-Glucosamine, 8% NaCl, D-Fructose, D-Fucose, D-Mannitol, D-Serine, L-Serine, Glucuronamide, Sodium Bromate and Tween 40. Negative tests with Biolog GEN III: Dextrin, Maltose, D-Trehalose, D-Cellobiose, Gentiobiose, D-Turanose, Stachyose, D-Raffinose, α-D-Lactose, D-Melibiose, β-Methyl-D-Glucoside, D-Salicin, *N*-Acetyl-D-Mannosamine, *N*-Acetyl-D-Galactosamine, *N*-Acetyl-Neuraminic Acid, 3-Methyl glucose, L-Fucose, L-Rhamnose, D-Sorbitol, D-Arabitol, myo-Inositol, D-Glucose-6-Phosphate, D-Fructose-6-Phosphate, D-Aspartic Acid, Gelatin, Glycl-L-Proline, L-Histidine, Pectin, D-Galacturonic Acid, L-Galactonic Acid Lactone, D-Glucuronic Acid, *p*-Hydroxyphenyl Acetic Acid, Methyl Pyruvate, D-Lactic Acid Methyl Ester, D-Malic Acid, Bromosuccinic Acid, α-Hydroxybutyric Acid, α-Ketobutyric Acid, Acetoacetic Acid, Formic Acid and Sodium Butyrate.
Country of origin	Serbia
Region of origin	Mionica, Western Serbia
Date of isolation	26/10/2021
Source of isolation	Rhizosphere of wheat (*Triticum aestivum* L.)
Sampling date	9/06/2021
Latitude	44.24759 N
Longitude	20.09931 E
Altitude	189 m
16S rRNA gene accession nr.	OP021715
Genome accession number	GenBank accession number: PRJNA859669
Genome status	Incomplete
Genome size	5,997,322 bp
GC mol%	60.4%
Number of strains in study	02
Source of isolation of non-type strains	Rhizosphere of wheat (*Triticum aestivum* L.)
Information related to the Nagoya Protocol	Implementation of Nagoya Protocol is still not fully in place in Serbia, Mr. Dusan Ognjanovic (Serbian representative for agreements on biological and genetic resources) was contacted regarding this issue.
Designation of the Type Strain	IT-P374^{T}
Strain Collection Numbers	CFBP 9061^{T}, LMG 32734^{T}, EML 1792^{T}

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interest or personal relationships that could have influenced the work reported in this paper.

ETHICAL STATEMENT

The experiments did not involve human participants and/or animals.

SUPPLEMENTARY DATA

Table S1. Origin of the *Pseudomonas* strains used in this study.

Isolate	Field location	GPS coordinates of the fields
IT-194P	MI 3	44.24540 N 20.10350 E
IT-215P	MI 4	44.24745 N 20.10012 E
IT-P366^T, IT-P374^T	MI 5	44.24759 N 20.09931 E

Table S2. Phenotypic characteristics of *Pseudomonas serbica* (with type strain IT-P366^T and the other strain IT-194P) and *Pseudomonas serboccidentalis* (with type strain IT-P374^T and the other strain IT-215P), determined by Biolog GEN III microplates. All of the strains were tested in triplicates. Literature data are shown for *Pseudomonas umsongensis* DSM16611^T (Furmanczyk et al., 2018) and *Pseudomonas koreensis* LMG21318^T (Morimoto et al., 2020).

Biolog GENIII	*P. umsongensis* DSM16611^T	IT-P366^T	IT-194P	*P. koreensis* LMG21318^T	IT-P374^T	IT-215P
Carbon sources						
Dextrin	-	w	-	-	-	-
Maltose	-	+	-	-	-	-
D-Trehalose	-	+	-	-	-	-
D-Cellobiose	-	-	-	-	-	-
Gentiobiose	-	-	-	-	-	-
Sucrose	-	+	-	-	+	+
D-Turanose	-	-	-	-	-	-
Stachyose	-	-	-	-	-	-
D-Raffinose	-	-	-	-	-	-
α-D-Lactose	-	-	-	-	-	-
D-Melibiose	-	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-	-
D-Salicin	-	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	+	-	+	w	w
N-Acetyl-D-Mannosamine	-	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	+	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-	-
α-D-Glucose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
D-Fructose	+	+	+	+	w	w
D-Galactose	+	-	+	+	+	+
3-Methyl glucose	-	-	-	+	-	-
D-Fucose	-	w	-	-	w	w
L-Fucose	-	-	-	-	-	-
L-Rhamnose	-	-	-	-	-	-
Inosine	-	w	-	-	+	+
D-Sorbitol	-	-	-	-	-	-
D-Mannitol	-	+	+	+	w	w
D-Arabitol	-	-	-	-	-	-
myo-Inositol	-	w	-	-	-	-
Glycerol	+	+	+	+	w	w
D-Glucose-6-Phosphate	-	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-	-
D-Aspartic Acid	-	-	-	-	-	-
D-Serine	-	+	-	-	w	w
Gelatin	-	+	-	-	-	-
Glycyl-L-Proline	+	+	-	-	-	-
L-Alanine	+	+	+	+	+	+

L-Arginine	+	+	+	+	+	+
L-Aspartic Acid	+	+	+	+	+	+
L-Glutamic Acid	+	+	+	+	+	+
L-Histidine	+	+	+	+	-	-
L-Pyroglutamic Acid	+	+	+	+	+	+
L-Serine	+	+	+	+	w	w
Pectin	+	-	-	-	-	-
D-Galacturonic Acid	+	-	-	-	-	-
L-Galactonic Acid Lactone	+	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+	+
D-Glucuronic Acid	+	-	+	-	-	-
Glucuronamide	+	w	w	-	w	w
Mucic Acid	+	+	+	+	+	+
Quinic Acid	+	+	+	+	+	+
D-Saccharic Acid	+	+	+	w	+	+
p-Hydroxyphenyl Acetic Acid	-	+	-	-	-	-
Methyl Pyruvate	+	w	w	-	-	-
D-Lactic Acid Methyl Ester	-	-	-	-	-	-
L-Lactic Acid	+	+	+	+	+	+
Citric Acid	+	+	+	+	+	+
α-Ketoglutaric Acid	+	+	+	-	+	+
D-Malic Acid	+	-	w	-	-	-
L-Malic Acid	+	+	+	+	+	+
Bromosuccinic Acid	+	w	w	-	-	-
Tween 40	+	w	w	-	w	w
γ-Amino-N-Butyric Acid	+	+	+	+	+	+
α-Hydroxybutyric Acid	+	-	-	-	-	-
β-Hydroxybutyric Acid	+	+	+	+	+	+
α-Ketobutyric Acid	-	-	-	-	-	-
Acetoacetic Acid	-	-	-	-	-	-
Propionic Acid	+	+	w	+	+	+
Acetic Acid	+	+	+	+	+	+
Formic Acid	+	w	w	-	-	-
Other Biolog Gen III tests						
pH 6.0	+	+	+	+	+	+
pH 5.0	+	+	+	+	+	+
1% NaCl	+	+	+	+	+	+
4% NaCl	+	+	+	+	+	+
8% NaCl	+	-	-	-	w	w
1% Sodium Lactate	+	+	+	+	+	+
Fusidic Acid	+	+	+	+	+	+
D-Serine	+	+	+	+	+	+
Troleandomycin	+	+	+	+	+	+
Rifamycin SV	+	+	+	+	+	+
Minocycline	-	+	-	-	+	+
Lincomycin	+	+	+	+	+	+
Guanidine hydrochloride	+	+	+	+	+	+
Niaproof 4	+	+	+	+	+	+
Vancomycin	+	+	+	+	+	+
Tetrazolium Violet	+	+	+	+	+	+
Tetrazolium Blue	+	+	+	+	+	+
Nalidixic Acid	+	+	-	+	+	+
Lithium Chloride	+	+	-	+	+	+
Potassium Tellurite	+	+	+	+	+	+
Aztreonam	+	+	+	+	+	+
Sodium Butyrate	-	+	-	-	-	-
Sodium Bromate	+	+	w	-	w	w

-, negative; +, positive; w, weak

Table S3. Strains of *Pseudomonas serbica* and *Pseudomonas serboccidentalis* and their zones of inhibition (mm) when grown in the presence of 10 different antibiotics, tested using the disc diffusion method (Bauer, 1966).

Antibiotics (amount per disk)	*P. serbica*		*P. serboccidentalis*	
	IT-P366^T	IT-194P	IT-P374^T	IT-215P
Imipenem (10 µg)	37.8	32.8	28.3	27.4
Ticarcillin (75 µg)	0	0	0	0
Meropenem (10 µg)	40.0	36.9	30.7	31.9
Ciprofloxacin (5 µg)	38.9	40.2	32.3	31.4
Cefepime (30 µg)	32.2	32.8	24.0	24.9
Ticarcillin / clavulanic acid (75+10 µg)	0	0	0	0
Aztreonam (30 µg)	14.2	0	12.1	14.2
Levofloxacin (5 µg)	29.8	34.1	24.3	26.9
Amikacin (30 µg)	32.8	32.0	25.9	25.9
Tobramycin (10 µg)	26.2	26.8	22.6	23.0

Table S4. Genomic features and accession numbers for all of the type strains used to construct *rrs* gene-based and whole-genome based phylogenetic trees (Figure 1 and Figure 2) retrieved from GenBank at the National Center for Biotechnology Information (NCBI) website. For *Pseudomonas helmanticensis*, strain BIGb0525 (from BioSample ID SAMN10361493) was used instead of the type strain because it is the only one available in the TYGS database for that species.

Preferred name	Deposit	Other deposits	Base pairs	Percent G+C	Bioproject accession	Biosample accession	Assembly accession	Reference
Pseudomonas fitomaticsae	FIT81	CECT 30374; DSM 112699	6,492,796	60.59	PRJNA224116	SAMN19241968	GCF_021018765	Atanasov et al., 2022
Pseudomonas baetica	LMG 25716	a390; CECT 7720	6,902,809	58.76	PRJNA303516	SAMN04488362	GCA_002813455	López et al., 2012
Pseudomonas laurylsulfatiphila	AP3_16	DSM 105097; PCM 2903	6,684,644	60.15	PRJNA390162	SAMN07222753	GCA_002934665	Furmanczyk et al., 2018
Pseudomonas reinekei	MT1	CCUG 53116; DSM 18361	6,249,573	59.17	PRJNA359931	SAMN06198581	GCA_001945365	Camara et al., 2007
Pseudomonas kribbensis	KCTC 32541	46-2; DSM 100278	6,324,282	60.55	PRJNA224116	SAMN09244908	GCF_003352185	Chang et al., 2016
Pseudomonas tensinigenes	ZA 5.3	CFBP 8882; LMG 32032	6,621,778	59.17	PRJNA639797	SAMN15248349	GCA_014268445	Girard et al., 2021
Pseudomonas iranensis	SWRI54	CFBP 8850; LMG 32039	6,073,514	59.89	PRJNA639797	SAMN15248341	GCA_014268585	Girard et al., 2022
Pseudomonas azerbaijanoccidentalis	SWRI74	CFBP 8868; LMG 32055	6,742,611	59.31	PRJNA639797	SAMN19473663	GCA_019145495	Girard et al., 2023
Pseudomonas azerbaijanorientalis	SWRI123	CFBP 8871; LMG 32058	6,228,218	60.11	PRJNA639797	SAMN19473666	GCA_019139795	Girard et al., 2024
Pseudomonas monsensis	PGSB 8459	CFBP 8854; LMG 32033	6,422,728	60.05	PRJNA639797	SAMN15248347	GCA_014268495	Girard et al., 2025
Pseudomonas zeae	OE 48.2	CFBP 8853; LMG 32031	6,630,739	58.99	PRJNA639797	SAMN15248348	GCA_014268485	Girard et al., 2026
Pseudomonas moorei	DSM 12647	CCUG 53114; RW10	6,546,438	59.66	PRJNA563568	SAMN04490195	GCF_900102045	Camara et al., 2007
Pseudomonas laurylsulfativorans	AP3_22	DSM 105098; PCM 2904	6,680,726	59.62	PRJNA369606	SAMN06289919	GCA_002906155	Furmanczyk et al., 2018
Pseudomonas moraviensis	LMG 24280	1B4; DSM 16007; JCM 14770; CCM 7280	6,092,441	60.17	PRJNA224116	SAMN04490196	GCF_900105805	Tvrzova et al., 2006
Pseudomonas koreensis	LMG 21318	LMG 21318; DSM 16610; JCM 14769; KACC 10848; Ps 9-14	6,123,813	60.53	PRJNA224116	SAMN04490189	GCF_900101415	Kwon et al., 2003
Pseudomonas silesiensis	A3	DSM 103370; PCM 2856	6,823,538	59.58	PRJNA315635	SAMN04566726	GCA_001661075	Kaminski et al., 2018
Pseudomonas atacamensis	M7D1	LMG 31516; CCUG 73898	6,170,631	59.88	PRJNA531338	SAMN11356701	GCA_004901935	Poblete-Morales et al., 2020
Pseudomonas umsongensis	DSM 16611	LMG 21317; KACC 10847; Ps 3-10	6,701,403	59.73	PRJNA390488	SAMN07236649	GCA_002236105	Kwon et al., 2003
Pseudomonas jessenii	DSM 17150	CIP 105274; ATCC 700870; CCUG 42059; CFML 95-307	6,537,206	59.7	PRJNA390484	SAMN07236640	GCA_002236115	Verhille et al., 1999
Pseudomonas glycinae	MS586	LMG 30275; NRRL B-65441	6,396,728	60.48	PRJNA309549	SAMN04435621	GCA_001594225	Jia et al., 2020
Pseudomonas mandelii	LMG 21607	CIP 105273; ATCC 700871; CCUG 42058; DSM 17967; JCM 21619	7,041,658	59.2	PRJEB16502	SAMN04489801	GCA_900106065	Verhille et al., 1999
Cellvibrio japonicus	Ueda107	NCIMB 10462; DSM 16015; NCDO 2697	4,576,573	51.99	PRJNA28329	SAMN02603448	GCA_000019225	Humphry et al., 2003
Pseudomonas rustica	DSM 112348	LMG 32241; MBT-4	5,854,577	58.92	PRJNA224116	SAMN18880036	GCF_018336155	Fiedler et al., 2022
Pseudomonas vancouverensis	LMG 20222	CIP 106707; ATCC 700688; CCUG 49675; DSM 17555; DhA-51	6,424,805	60.11	PRJNA224116	SAMN05216558	GCF_900105825	Mohn et al., 1999
Pseudomonas izuensis	IzPS43_3003	LMG 31527; CECT 9963	6,857,708	59.62	PRJNA594796	SAMN13530344	GCA_009861505	Lu et al., 2020
Pseudomonas neuropathica	P155	CCUG 74875; CECT 30178	6,471,826	59.23	PRJNA224116	SAMN15848192	GCF_015461835	Duman et al., 2019
Pseudomonas crudilactis	UCMA 17988	LMG 31804; DSM 109949	6,626,596	59.13	PRJNA224116	SAMN13762321	GCF_013433315	Schlusselhuber et al., 2021
Pseudomonas botevensis	COW3	CFBP 8873; LMG 32176	6,448,664	61.21	PRJNA639797	SAMN19473668	GCA_019145475	Girard et al., 2021
Pseudomonas germanica	FIT28	LMG 32353; DSM 112698	6,713,530	59.09	PRJNA705867	SAMN18105273	GCA_019614655.1	Atanasov et al., 2022
Pseudomonas mohnii	DSM 18327	CCUG 53115; IpA-2	6,592,588	59.62	PRJEB16418	SAMN05216205	GCA_900105115	Camara et al., 2007
Pseudomonas azotifigens	LMG 31526	CECT 9962; IzPS32d	6,563,527	60.31	PRJNA665284	SAMN16250066	GCA_014863585	Morimoto et al., 2021
Pseudomonas allokribbensis	LMG31525	CECT 9961; IzPS23	6,565,027	60.34	PRJNA665319	SAMN16250065	GCA_014863605	Morimoto et al., 2021
Pseudomonas granadensis	LMG 27940	DSM 28040; F-278; F-278,770	5,943,070	60.16	PRJEB16372	SAMN05216579	GCA_900105485	Pascual et al., 2015
Pseudomonas mucoides	P154a	CCUG 74874; CECT 30177	7,035,943	58.84	PRJNA224116	SAMN15848106	GCF_015461845	Duman et al., 2019
Pseudomonas piscis	KCTC 72033	MCCC 1K03575; MC042	6,921,954	63.57	PRJNA578493	SAMN13064563	GCA_009380155	Liu et al., 2020
Pseudomonas rhizosphaerae	DSM 16299	LMG 21640; CECT 5726; IH5	4,688,635	61.99	PRJNA261876	SAMN03077633	GCA_000761155	Peix et al., 2003
Pseudomonas lutea	DSM 17257	LMG 21974; CECT 5822; OK2	5,647,497	60.15	PRJNA261881	SAMN03077637	GCA_000759445	Peix et al., 2004
Pseudomonas abietaniphila	ATCC 700689	CIP 106708; CCUG 50779; DSM 17554; BKME-9	7,222,451	59.44	PRJEB15899	SAMN05216605	GCA_900100795	Mohn et al., 1999
Pseudomonas kuykendallii	NRRL B-59562	LMG 26364; DSM 25107; H2	4,689,718	65.9	PRJEB16580	SAMN05216287	GCA_900106975	Hunter et al., 2012
Pseudomonas graminis	DSM 11363	CIP 105897; CCUG 51504; P 294/08	5,845,484	60.26	PRJEB17037	SAMN05216197	GCA_900111735	Behrendt et al., 1999
Pseudomonas psicuculturae	P115	CCUG 74873; CECT 30173	6,218,846	60.03	PRJNA224116	SAMN16090520	GCF_015461805	Duman et al., 2019
Pseudomonas baltica	MBT-2	LMG 31955; DSM 111761	4,573,010	62.49	PRJNA224116	SAMN15808892	GCF_014235765	Gieschler et al., 2019
Pseudomonas atagonensis	PS14	LMG 31496; CECT 9940	6,115,729	59.62	PRJNA565185	SAMN12736367	GCA_011369485	Morimoto et al., 2020
Pseudomonas sichuanensis	WCHPS060039	CNCTC 7662; GDMCC 1.1424	6,018,116	63.87	PRJNA415331	SAMN09302769	GCA_003231305	Qin et al., 2019
Pseudomonas marginalis	DSM 13124	CFBP 1387; CFBP 2037; LMG 2210; LMG 2215; CIP 106712	7,182,085	60.63	PRJNA548434	SAMN12033583	GCA_007858155	Stevens, 1925
Pseudomonas akappageensis	PS24	LMG 31497; CECT 9941	6,365,731	60.21	PRJNA565332	SAMN12740357	GCA_011355085	Morimoto et al., 2020
Pseudomonas piscicola	P50	CCUG 74871; CECT 30175	6,900,506	58.5	PRJNA657457	SAMN15829822	GCA_015351605	Duman et al., 2019
Pseudomonas juntendi	BML3	DSM 109244; JCM 33395	5,731,217	62.66	PRJDB8113	SAMD00166173	GCA_009932375.1	Tohya et al., 2019
Pseudomonas helmanticensis	BIGb0525		6,628,093	59.1	PRJNA501417	SAMN10361493	GCA_004365765.1	Ramirez-Bahena et al., 2014

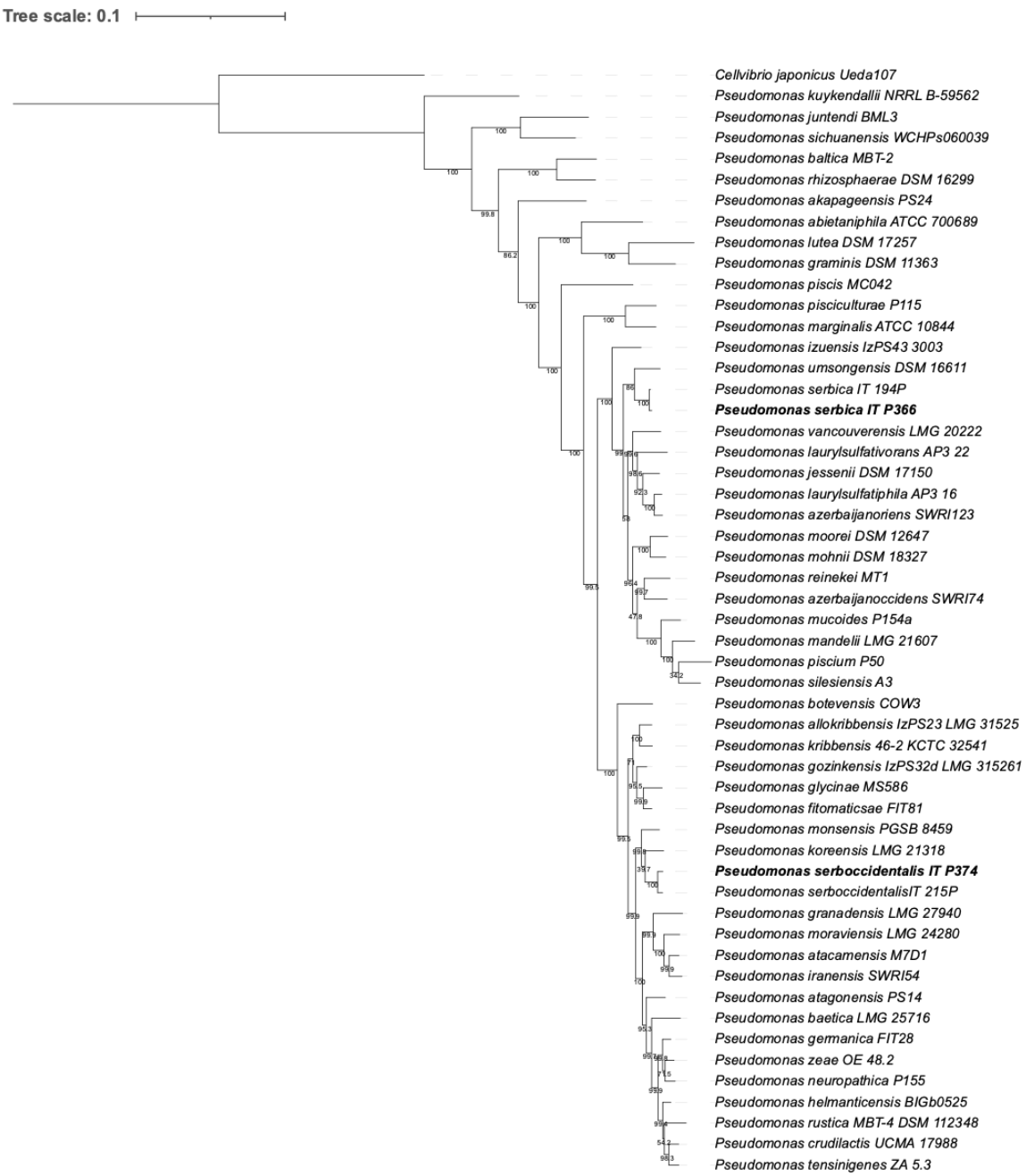

Figure S1. Phylogenetic analysis based on the concatenated sequences of genes *rrs* (16S rRNA gene, 1549 bp), *gyrB* (2443 bp), *rpoB* (4091 bp) and *rpoD* (1891 bp). Sequences were aligned with Muscle5 v.3.8.31 (Edgar, 2021) and the alignment was subsequently used for reconstruction of the phylogenetic tree based on maximum likelihood method with GTR+F as substitution model, using the software IQ-TREE v.2.2.0.3 (Minh et al., 2022). Numbers at nodes are bootstrap values shown as percentages of SH-like aLRT with 1000 replicates.

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PART B

Fluorescent *Pseudomonas* from suppressive and non-suppressive soils share genomic and functional traits

FLUORESCENT *PSEUDOMONAS* FROM SUPPRESSIVE AND NON-SUPPRESSIVE SOILS SHARE GENOMIC AND FUNCTIONAL TRAITS

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ABSTRACT

Suppressive soils have gained attention for their ability to maintain plant health despite the presence of pathogens. This suppressiveness is often attributed to the activities of the soil microbiota, including fluorescent *Pseudomonas* strains that exhibit biocontrol mechanisms. In contrast, conducive soils lack biocontrol activity but also harbor fluorescent *Pseudomonas* populations. In this study, we aimed to investigate whether there are differences in the fluorescent *Pseudomonas* community between suppressive and conducive soils, and whether these differences are reflected in their genome properties. To accomplish this, we compared the *Pseudomonas* communities in suppressive and conducive soils infected with *Fusarium graminearum* near Mionica, Serbia. Through *rpoD* metabarcoding, we assessed the composition of the *Pseudomonas* community and isolated strains from the rhizospheres of wheat plants cultivated in these soils. Our results showed that suppressive soils had higher richness and relative abundance of fluorescent *Pseudomonas* compared to conducive soils. However, genomic comparisons and *in vitro* assays revealed that the isolated *Pseudomonas* strains from both soil types exhibited similar capacities. In conclusion, we found that *Pseudomonas* species in both suppressive and conducive soils might display similar biocontrol functions.

Keywords: phytopathogens, PGPR, diversity, biocontrol agents, comparative genomics

INTRODUCTION

Suppressive soils are those where a low incidence of plant disease is observed despite a susceptible host plant, a virulent pathogen and favorable abiotic conditions for disease development (Cook & Rovira, 1976; Weller et al., 2002). This specific suppressiveness may add to soil fungistasis (or mycostasis), which is described as the ability of soils to inhibit fungal growth and spore germination (Termorshuizen & Jeger, 2008; Garbeva et al., 2011; Sipilä et al., 2012), resulting into pathogen suppression. Suppressive and fungistatic soils entail microbial populations with phytoprotective capacity, which have the ability of restricting the pathogen growth or/and survival. Besides, bacteria from suppressive soils might also contribute indirectly to disease suppression, by triggering induced systemic resistance (ISR) in plants (Tamietti & Matta, 1984; Tamietti et al., 1993; Lv et al., 2023).

Among phytoprotective microorganisms, *Pseudomonas* species hold an important place, as they have an important role in rhizosphere functioning and may contribute to pathogen and disease suppression through different modes of action (Weller et al., 2007; Kyselková & Moënne-Loccoz, 2012). Indeed, certain *Pseudomonas* produce antimicrobial secondary metabolites, such as pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), phenazine, or hydrogen cyanide (HCN), as well as lytic enzymes with biocontrol potential, such as chitinases or cellulases (Loper et al., 2012; Sarma et al., 2014; Vacheron et al., 2016; Kumar et al., 2017), which could directly inhibit pathogens. They may also elicit ISR in plants by producing lipopolysaccharides or flagella, DAPG or siderophores (Bakker et al., 2007). *Pseudomonas* with biocontrol properties, isolated from soils suppressive to take-all disease of wheat or barley, caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Cook & Rovira, 1976) or soils suppressive to *Thielaviopsis basicola*-mediated black root rot of tobacco (Stutz et al., 1986) effectively protected plants from disease (Almario et al., 2014a). Ecological and genomic characterization of biocontrol *Pseudomonas* is important to understand microbial phytoprotection (Smits et al., 2019; Zboralski et al., 2023), and we hypothesize that implementation of this approach to rhizosphere pseudomonads from suppressive and conducive soils may provide insight into the mechanisms potentially contributing to soil suppressiveness, all the more as *Pseudomonas* strains with biocontrol potential can also occur in conducive soils (Ramette et al., 2006; Frapolli et al., 2010).

The objective of this work was to identify the genomic and functional particularities of *Pseudomonas* bacteria in suppressive vs. non-suppressive soils. To this end, we chose suppressiveness to damping-off caused by *Fusarium graminearum*, a mycotoxin-producing, ascomycetous fungi causing damping-off, crown and root rot and Fusarium head blight (FHB or scab) on wheat (Besset-Manzoni et al., 2019), because (i) *Pseudomonas* strains may protect plants from *Fusarium* diseases (Almario et al., 2014b; Hu et al., 2014) and play a role in soil suppressiveness to these diseases (Kloepper et al., 1980; Alabouvette, 1999; Mazurier et al., 2009; Kyselková & Moënne-Loccoz, 2012), (ii) soils suppressive or conducive to Fusarium damping-off occur side by side near Mionica (Serbia), and (iii) the fungistatic or non-fungistatic status of these soils with regards to *F. graminearum* is also documented (Todorović et al., submitted; Chapter 2). The diversity of fluorescent *Pseudomonas* in the rhizosphere of wheat grown in these soils was analyzed through a metabarcoding approach targeting the *rpoD* gene of the *P. fluorescens* group, *Pseudomonas* isolates were obtained and characterized based on genomic and functional traits.

MATERIAL AND METHODS

Fusarium graminearum fungal strain and preparation of spore suspension

The highly virulent and toxin-producing *Fusarium graminearum* MDC_Fg1 isolate (hereafter termed *F. graminearum* Fg1) used in the experiments was isolated from naturally infected cereal grains in northern France (Alouane et al., 2018). Spore suspension of *F. graminearum* Fg1 was prepared by growing the fungus in Mung Bean Broth (MBB) (Evans et al., 2000) for 6 days at 22°C, with shaking at 180 rpm (Incubator Shaker Series I26, New Brunswick Scientific Co., Inc., Edison, NJ, USA). After incubation, a volume of the preculture was taken and diluted to one tenth in fresh MBB and incubated for 10 days under the same conditions. The resulting culture was vortexed, filtered to discard mycelium and centrifuged at 4700*xg* for 10 min (Avanti J-E Series, Beckman Coulter, Fullerton, CA, USA). Supernatant was discarded and the resulting pellet was washed twice with sterile water. Titration of spores in the suspension was estimated using a Thoma counting chamber.

Isolation of *Pseudomonas*

Soils MI2, MI3, MI4 and MI5 located near Mionica, Serbia (Table 1) were chosen for isolation of *Pseudomonas*. These soils were already tested for fungistasis and suppressiveness properties towards *F. graminearum*-mediated damping-off (Todorović et al., submitted; Chapter 2). Based on fungistasis and suppressiveness properties of these four soils, they were categorized in three soil categories: (i) soils MI2 and MI3 (reffered to as MI2/MI3 here) were fungistatic and suppressive, (ii) soil MI4 was non-fungistatic and non-suppressive, while (iii) soil MI5 was non-fungistatic and suppressive.

Table 1. Origin of the soils from Mionica where *Pseudomonas* strains were isolated. Results of fungistasis to *Fusarium graminearum* Fg1 and suppressiveness to *Fusarium graminearum* Fg1 disease in wheat were from Todorović et al. (submitted; Chapter 2).

Field	GPS coordinates	Manure amendments	Fungistasis to *F. graminearum* Fg1	Soil suppressiveness to *F. graminearum* Fg1 disease
MI2	44.24611 N 20.10431 E	With manure	Yes	Yes
MI3	44.24540 N 20.10350 E	With manure	Yes	Yes
MI4	44.24745 N 20.10012 E	No manure	No	No
MI5	44.24759 N 20.09931 E	No manure	No	Yes

Half of the seeds of winter wheat (*Triticum aestivum* L.) variety Récital were inoculated with spore suspension of *F. graminearum* Fg1, whereas the other half was not inoculated, and seeds were grown in the four MI soils for 28 days. Whole plants were harvested and vigorously shaken to dislodge loosely-adhering soil, and roots and closely-adhering soil were used as rhizosphere samples, following a protocol adapted from Bulgarelli et al. (2012). For each soil and plant condition [i.e., 4 soils × 2 conditions (seeds inoculated and non-inoculated with *F. graminearum* Fg1)], wheat root systems with adhering soil were put in 50 mL of phosphate buffered saline (NaCl, 8 g; KCl, 0.2 g; KH_2PO_4, 0.24 g; Na_2HPO_4, 1.42 g; H_2O, 1000 mL) and shaken for one hour at 160 rpm. The roots were discarded, the suspension was centrifuged at 4000*xg* for 20 min, after which the supernatant was discarded. The pellet was resuspended in 20 mL 0.8% NaCl and represented the rhizosphere soil extract.

Isolation of *Pseudomonas* was done in 96-well microplates, using the rhizosphere extracts of inoculated and non-inoculated soils MI2, MI3, MI4 and MI5 [i.e., 4 soils × 2 (inoculated/not with *F. graminearum* Fg1)], according to Vacheron et al. (2016). In brief, 20

µL of each rhizosphere extract was mixed with 180 µL of King's B^{+++} [i.e., King's B (Condalab, Madrid, Spain) supplemented with ampicillin (40 $\mu g.mL^{-1}$), chloramphenicol (13 $\mu g.mL^{-1}$) and cycloheximide (100 $\mu g.mL^{-1}$); McSpadden Gardener et al., 2000] and transferred into each of five microplate wells. The suspensions were serially diluted, following a most probable number (MPN) design with five wells per dilution. The microplates were incubated at 28°C for 24 h, and then 1 µL from each last positive well was plated on King's B agar (Condalab). After growth, at least 48 isolates were randomly picked for each condition and all were purified three times consecutively.

rpoD analysis of rhizosphere *Pseudomonas*

The non-inoculated plants (six rhizosphere replicates per soil x condition) harvested at 28 days were also used to assess root-associated *Pseudomonas* populations. Each root system was shaken to dislodge loosely-adhering soil and was flash-frozen in liquid nitrogen, followed by lyophilisation (24 h, at −50°C). The root-adhering soil (i.e., rhizosphere soil) was separated from the roots using brushes and stored at −80°C, prior to DNA extraction using the FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch-Graffenstaden, France). DNA was extracted and eluted in 50 µL sterile ultra-pure water, according to the manufacturer's instructions, and DNA concentrations were determined using a UV Spectrophotometer (NanoPhotometer NP80, Implen, Munich, Germany).

The *rpoD* gene coding for RNA polymerase sigma 70 (sigma D) factor was chosen to visualize diversity within the *Pseudomonas* genus. Primers with specific Illumina tails (rpoD_F: TCGCCAAGAAGTACACCAAC and rpoD_R: CCATGGAGATCGGCTCTT) (Manriquez, 2021) were designed to amplify a 356 bp fragment of *rpoD*. PCR was done under the following conditions: 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 59°C for 40 s and 72°C for 45 s, with a final elongation step at 72°C for 10 min. PCR product purification, amplicon library construction, and Illumina MiSeq sequencing (2 × 300 bp paired-end reads) were performed by Microsynth (Vaulx-en-Velin, France). Total reads obtained were demultiplexed. Reads quality was assessed using the software fastp v.0.23.2 (Chen et al., 2018) and primers were removed using the software cutadapt v.4.1 (Martin, 2011) with the default parameters. Then, the sequencing paired-end reads were processed using R software v.4.2.1 and the DADA2 package v.1.26 (Callahan et al., 2016) through a workflow step including filtering, trimming, denoising, dereplicating, merging and finally chimera removing. In the end, 928,217 reads were kept and distributed in 823 amplicon sequence variants (ASVs). Next, taxonomy was assigned using the DADA2 native implementation of the naïve Bayesian classifier method (Wang et al., 2007) and a home-made *rpoD* sequence database specific to the primer pairs. In conclusion, 43 genera in total were identified in the microbial community, with 41 ASVs belonging to the *Pseudomonas* genus.

Identification of *Pseudomonas*

Genomic DNA of all the isolated *Pseudomonas* was extracted from overnight cultures in Tryptic Soy Broth (TSB; Carl Roth, Karlsruhe, Germany), using NucleoSpin R 96 Tissue kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Isolate characterization was performed by sequencing *rpoD* gene, using primers rpoDf (5'-ACTTCCCTGGCACGGTTGACCA-3') and rpoDr (5'-TCGACATGCGACGGTTGATGTC-3') (Frapolli et al., 2007). When *rpoD* amplification did not succeed, the *rrs* gene was amplified with primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards et al., 1989) and sequenced. Each PCR reaction was done in a volume of 50 µL, which contained 5 µL of 10 × DreamTaq Green Buffer (Thermo Fisher Scientific, Waltham, MA, USA), 5 µL of dNTP (2 mM) (Thermo Fisher Scientific), 0.25 µL of DreamTaq DNA polymerase

(5 U.μL^{-1}), 2.5 μL of each primer (10 μM), 50 ng of DNA, and RNase-free water up to 50 μL. Reaction conditions for primer pair rpoDr/rpoDf were 94°C for 150 s, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final elongation step at 72°C for 10 min, and for pA/pH 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 35 s, with a final elongation step at 72°C for 3 min. Amplified fragments were sequenced with Sanger sequencing at Microsynth, in forward direction. Isolates were identified to the genus level using the GenBank database and BLAST option (based on hits with very high query coverage and percent identity).

Analysis of sequences was done using the SeaView multiplatform (Gouy et al., 2010). The sequences were aligned with Muscle5 v.3.8.31 (Edgar, 2022) and they were manually filtered to discard gaps and aligned regions of low quality. Gblock software (Castresana, 2000; Talavera & Castresana, 2007) was used to eliminate poorly aligned positions as well as divergent regions to prepare for phylogenetic analysis, and all duplicated sequences were discarded with seqkit software (Shen et al., 2016). The phylogenetic tree was constructed with Distance method and 1000 bootstraps and visualized using iTol (Letunic & Bork, 2021).

DNA extraction, genome sequencing and assembling

DNA extraction for genome sequencing was done from an overnight TSB culture, using a Nucleospin tissue kit (Macherey-Nagel), according to the manufacturer's instructions. Genomic DNA library preparation and sequencing were done at Novogene (Cambridge, England), using Illumina NovaSeq 6000 technology. Genomic DNA was randomly sheared into short fragments. The obtained fragments were end repaired, A-tailed and further ligated with Illumina adapter, generating a 2 × 150-bp paired-end library. The fragments with adapters were PCR amplified, size selected, purified and sequenced. The original data from Illumina platform were recorded in a FASTQ file, which contains sequencing reads and sequencing quality information. fastp software v.0.23.1 (Chen et al., 2018) with default settings was used for trimming sequences and Unicycler software v.0.5.0 (Wick et al., 2017) with default settings for *de novo* assembly. Identification and construction of phylogenetic tree was performed with the Type strain Genome Server (TYGS) (https://tygs.dsmz.de/; Meier-Kolthoff & Göker, 2019; Meier-Kolthoff et al., 2022). Genomic features of the isolates were obtained using the MicroScope platform (Vallenet et al., 2020).

Genome annotation

Genome annotation was done automatically with the MicroScope platform (v.3.15.4; Vallenet et al., 2020). DIAMOND blastp (v.2.0.8.146; Buchfink et al., 2015), was used to search for genes involved in biocontrol and plant-growth promotion (accession numbers are available in Chapter 3; Table S1) within genome protein sequences using the options --query-cover 80 --id 70, in order to filter the hits with minimum 80% query coverage and minimum 70% amino acid identity.

The searched functions included (i) production of antimicrobial compounds phenazine (*phzABCDEFG*) (Dar et al., 2020), 2-hexyl-5-propyl-alkylresorcinol (HPR) (*darABC*) (Nowak-Thompson et al., 2003), DAPG (*phlABCD*) (Bangera & Thomashow, 1999), pyrrolnitrin (*prnABCD*) (Kirner et al., 1998), HCN (*hcnABC*) (Ramette et al., 2003) and pyoluteorin (*pltABCDEFGLM*) (Nowak-Thompson et al., 1999), (ii) production of insect toxin FitD (*fitD*) (Loper et al., 2012) and alkaline metalloproteinase AprA (*aprA*) (Loper et al., 2012) involved in biocontrol, (iii) production of siderophores pyoverdine (*pvdL*) (Schalk & Guillon, 2013), pyochelin (*pchABCDEF*) (Reimmann et al., 2001) and pseudomonine (*pmsABCE*) (Matthijs et al., 2009), (iv) signaling and modulation of plant hormonal balance by deamination of ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (*acdS*) (Shah et al., 1998),

ethylene production (*efe*) (Wang et al., 2010), auxin biosynthesis (*iaaMH*, *ipdC*/*ppdC*) (Loper et al., 2012; Cécile Gruet et al., 2022), auxin catabolism (*iacABCDEFGHI*) (Loper et al., 2012), acetoin synthesis (*budB*/*ilvNB*/*alsS*, *budA*/*alsD*) (Blomqvist et al., 1993; Loper et al., 2012), 2,3-butanediol synthesis (*budC*/*ydjL* in addition to the acetoin synthesis genes) (Nicholson, 2008), 2,3-butanediol conversion to acetoin (*adh*/*bdhA*/*ydjL*) (Huang et al., 1994; Nicholson, 2008), acetoin catabolism (*acoABCX*) (Huang et al., 1994), (v) transformation of P and N sources by phosphate solubilization (*gcd*, *gad*) (Miller et al., 2010), nitrogen fixation (*nifHDK*) (Bruto et al., 2014) and denitrification (*nirK*, *nirS*) (Bruto et al., 2014; Coyne et al., 1989). In case where presence of more than one gene is necessary to achieve a function (*e.g.*, presence of both *iaaM* and *iaaH* for the synthesis of auxin via the indole-3-acetamide pathway) but only some of the necessary genes were found in the genome, we checked for the presence of the missing genes with less stringent BLAST result filtering criteria (--query-cover 80 --id 30). Putative biosynthetic gene clusters were further identified using antiSMASH (Blin et al., 2019) within the MicroScope platform and the annotations were manually curated.

Carbohydrate-active enzymes (CAZymes) were predicted using dbCAN2 v.3 (Zhang et al., 2018) and compared with the CAZy database using HMMER v.3.3 (Eddy, 2011). Prediction of function and substrate specificity of CAZyme families or subfamilies was performed based on a review of activities assigned to CAZymes with known structures (characterized enzymes) in the CAZy database (http://www.cazy.org) (Lombard et al., 2014) and manually curated, as previously described (López-Mondéjar et al., 2022). A heatmap based on CAZyme counts was generated by pheatmap v.1.0.12 package (Kolde, 2019).

Phenotypic characterization of *Pseudomonas*

Screening of isolates for plant growth-promoting and biocontrol properties targeted production of HCN (Bakker & Schippers, 1987) and production of lytic enzymes, i.e., extracellular protease on milk agar, chitinase on minimal medium (Kim et al., 2003) supplemented with 10% colloidal chitin solution (prepared as described by Murthy & Bleakley, 2012), and cellulase on medium containing Carboxymethyl cellulose (CMC; Sigma Aldrich, USA) (Teather & Wood, 1982; Chantarasiri, 2014). The ability of isolates to solubilize inorganic P sources was tested on NBRIP medium (National Botanical Research Institute's Phosphate; Nautiyal, 1999), and their ability to produce siderophores was tested according to a protocol by Pérez-Miranda et al. (2007). Screening also included testing of metabolites involved in modulation of plant hormonal balance, such as ACC deaminase activity, tested according to a protocol by Penrose & Glick (2003) that detects α-ketobutyrate production. Screening for production of (i) seven auxin phytohormones, i.e., indole-3-acetic acid (IAA), indole-3-lactic acid, indole-3-carboxylic acid, indole-3-pyruvic acid, indol-3-butyric acid (IBA), tryptophol and indole-3-propionic acid, (ii) five cytokinins, i.e., trans-zeatin, trans-zeatin riboside (ZR), kinetin, 6-benzylaminopurine (BAP) and isopentenyl adenosine (IPA), (iii) two gibberellins, i.e., gibberellin A1 (GA1) and gibberellic acid (GA3), (iv) abscisic acid (ABA) and (v) kynurenic acid was done by Ultra High Performance Liquid Chromatography (UHPLC). Briefly, all isolates were grown 3 days at 28°C (300 rpm) in 2 mL of M9 minimal medium (Miller, 1972) supplemented with 0.4 mM of tryptophan and 0.1 mM of adenine. The cultures were centrifuged at 4500*xg* during 8 min and filtered at 0.2 µm. Supernatants were lyophilized (Alpha 1–4 LSC Martin Christ, Osterode, Germany) for 24 h, the powder obtained was extracted two times with methanol, drying with speed-vac (Centrivap Cold Trap Concentrator LABCONCO, Kansas City, MO, USA), and UHPLC separation was performed with an Agilent 1290 Series instrument (Agilent Technologies France, Les Ulis, France) using a 100 × 3 mm reverse phase column (Agilent Poroshell 120 EC-C18, 2.7 µm particle size). Samples (3 µL) were loaded onto the column equilibrated with solvent A (water + 0.4% formic acid) and solvent B (acetonitrile) in a 98:2 ratio. Compounds were eluted by increasing the

acetonitrile concentration to 40% over a 6 min period, then to 100% over 4 min, followed by an isocratic step of 2 min, at a flow rate of 0.5 $mL.min^{-1}$. Hormones were detected with a diode array detector (DAD) and an Agilent 6530 Q-TOF mass spectrometer in positive and negative electrospray ionization, based on comparison with commercial standards on both mass and UV (between 190 and 600 nm) chromatograms, along with accurate mass and UV spectra.

Inhibitory effect of *Pseudomonas* isolates towards *Fusarium graminearum*

The inhibitory effect of Volatile Organic Compounds (VOCs) produced by rhizosphere isolates towards *F. graminearum* Fg1 was assessed in a system of two Petri dishes sealed together with parafilm. For this assay, 30 µL of each bacterial suspension of optical density 1 (OD 600 nm) (Ultrospec 10 Cell Density Meter; Amersham Biosciences, Little Chalfont, UK) was spread onto Tryptone Soya Agar (TSA; Carl Roth). Plates containing Potato Dextrose Agar (PDA; Condalab) were center-inoculated with discs (Ø7 mm) taken from the edges of 8-day-old *F. graminearum* Fg1 colonies. After 24 h of bacterial and fungal growth, at 28°C and 22°C, respectively, the lid of TSA plate with bacteria was replaced with a plate containing *F. graminearum* Fg1 and the two plates were firmly sealed together with parafilm. Control plates were prepared in the same way, but without the bacteria in the bottom plate. The sealed plates were incubated at 22°C, and the observations were recorded at 72 h. Mycelial growth inhibition (%) of the fungus was determined according to Trivedi et al. (2008), using the formula $(1 - r_2/r_1) \times 100$, which considers radial growth of *F. graminearum* Fg1 in control plates (r_1) and plates with bacteria (r_2).

The effect of bacterial isolates on spore germination of *F. graminearum* Fg1 was tested in a microplate, according to Besset-Manzoni et al. (2019). The supernatant of each isolate was prepared from an overnight TSB culture and filtered at 0.2 µm. *F. graminearum* Fg1 spore suspension (10^4 $spores.mL^{-1}$) was prepared by growing the fungus in MBB, as described above. For each assay (in triplicate), 100 µL of bacterial supernatant, 100 µL of Potato Dextrose Broth (PDB; Condalab) and 50 µL of spore suspension were added per microplate well. For positive control, 100 µL of TSB was used instead of bacterial supernatant, and for negative control, 50 µL of PDB was used instead of spore suspension. After incubating microplates for 5 days at 28°C, the turbidimetry was measured at 492 nm using an Infinite M200 Pro microplate reader (TECAN, Mannedorf, Switzerland), and the value of the negative control was subtracted from that of each bacterial treatment and compared with that of the positive control.

Statistical analyses

All statistical analyses were performed using the R software v.4.2.1. (https://www.r-project.org), at $P < 0.05$. For MPN calculations, for each soil condition (soil inoculated or not with *F. graminearum* Fg1), data were log-transformed for normal distribution and variance homogeneity, and Student's *t* tests were performed to assess inoculation effect. Results are presented as means ± standard deviation. For *rpoD* microbiota analysis, the packages phyloseq (McMurdie & Holmes, 2013), vegan (Dixon, 2003) and ade4 (Dray & Dufour, 2007) were used. Alpha diversity analysis was performed by computing the index of observed richness and Chao1 for richness (Chao, 1987), and Shannon (Shannon, 1948) and inverse Simpson (Simpson, 1949) for diversity and evenness. Relationships between soil and the presence/absence of *Pseudomonas* were evaluated using the envfit procedure of the package vegan. Graphs and figures were plotted using the package ggplot2 (Wickham, 2011).

RESULTS

Enumeration of fluorescent *Pseudomonas* in the rhizosphere

Using the MPN method, the number of culturable putative fluorescent *Pseudomonas* ranged from 2.7×10^7 cells/root system for inoculated MI5 soil to 8.0×10^9 cells/root system for non-inoculated MI5 soil (Table S1). Overall, levels were higher (P = 0.046; Figure S1) for non-inoculated soils (9.18 ± 0.85 log cells/root system) than soils inoculated with *F. graminearum* Fg1 (7.92 ± 0.53 log cells/root system).

Microbiota diversity analyzed through *rpoD* metabarcoding

When *rpoD* metabarcoding was done for each of the MI2/MI3 (fungistatic and suppressive), MI4 (non-fungistatic and non-suppressive) and MI5 (non-fungistatic and suppressive) soil categories (Figure S2), the differences in observed richness, Chao1 and Shannon indices between the three soil categories MI2/MI3, MI4 and MI5 were not significant, but the inverse Simpson index in soil MI4 differed from those in soils MI2/MI3 and MI5 (P < 0.05) (Figure 1). The *Pseudomonas* subcommunity consisted of 4 to 12 species, depending on the soil sample, and its genotypic profile varied from one soil to the next, even within the MI2/MI3 soil category (Figure 2). In summary, the *Pseudomonas* subcommunity differed between the individual soils.

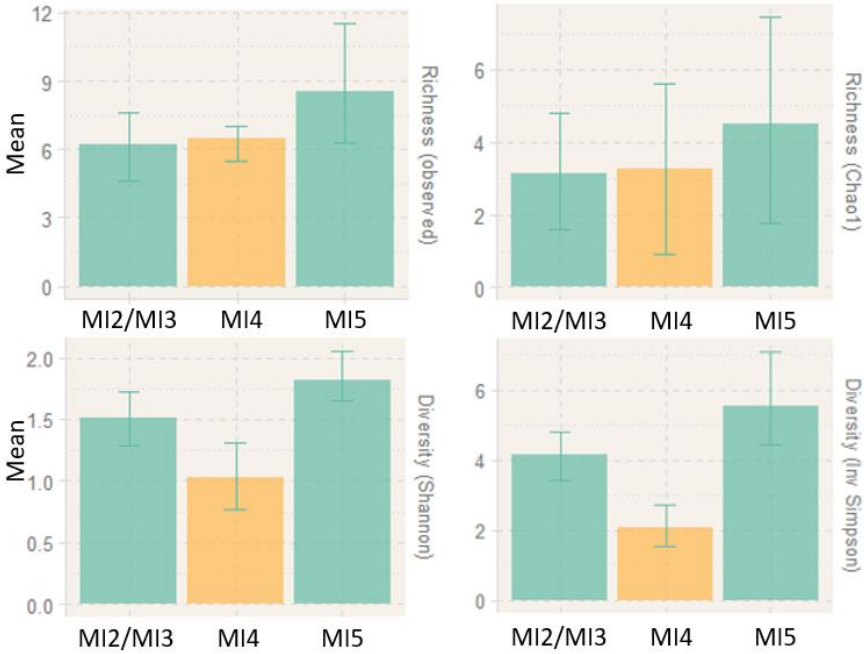


Figure 1. α-Diversity (boxplots) of *Pseudomonas* communities in MI2/MI3 (fungistatic and suppressive), MI4 (non-fungistatic and non-suppressive) and MI5 (non-fungistatic and suppressive) soil categories. Vertical bars represent 95% confidence intervals of the mean. Diversity indices for MI2/MI3 category were calculated from the MI2 and MI3 soils metabarcoding data treated together.

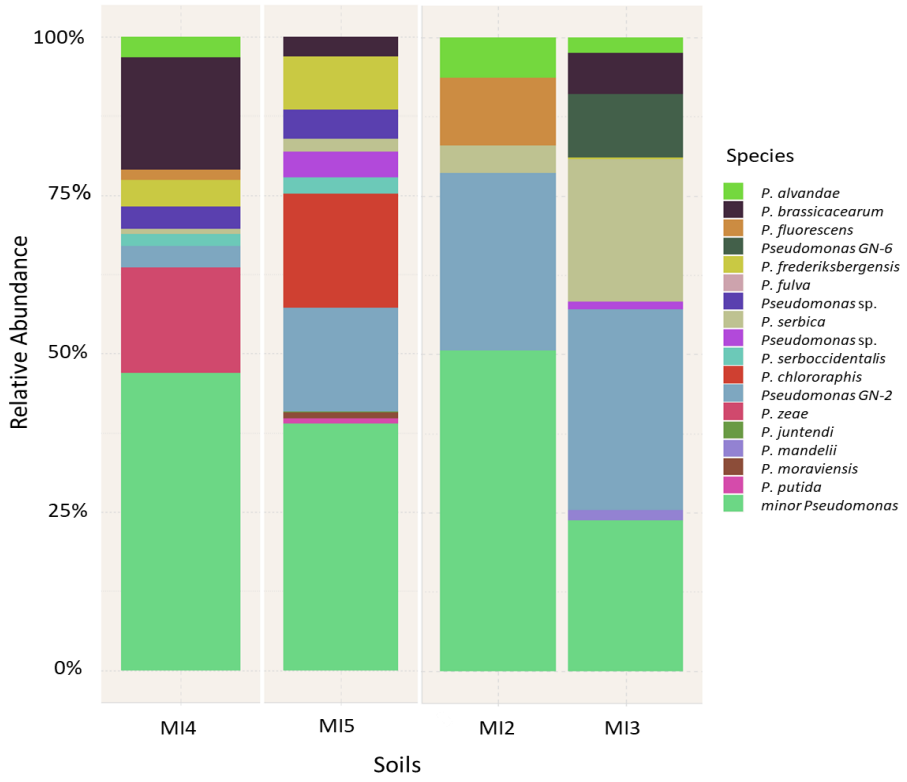


Figure 2. Relative abundance (sequence %) of different *Pseudomonas* species in soils MI4, MI5, MI2 and MI3. Species with relative abundance of < 1% were included into 'minor *Pseudomonas*'. Different *Pseudomonas* sp. represent different species based on *rpoD* gene sequence.

Taxonomic characterization of *Pseudomonas* isolates

A total of 406 putative *Pseudomonas* isolates were obtained, and the use of *rpoD* primers specific for the *P. fluorescens* group was successful for 185 of them, yielding 65 different *rpoD* sequences (Figure S3). *rpoD*-sequenced isolates belonged to 7 out of 11 subgroups of the *P. fluorescens* group that are outlined in Girard et al. (2021), i.e., the subgroups *P. fluorescens*, *P. kielensis*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata* and *P. chlororaphis*, while none of the isolates belonged to the subgroups *P. protegens*, *P. asplenii*, *P. gessardii* or *P. fragi* (Figure S3). Gblock (Castresana, 2000; Talavera & Castresana, 2007) and seqkit (Shen et al., 2016) were used to identify one isolate for each of the 65 *rpoD* sequences, and 29 of them (16 from inoculated wheat and 13 from non-inoculated wheat) were chosen for genome sequencing, i.e., 8 from soil MI2, 5 from soil MI3, 9 from soil MI4 and 7 from soil MI5 (Figure 3). The putative *Pseudomonas* isolates not amenable to *rpoD* sequencing were characterized by sequencing the 16S rRNA gene *rrs* (Figure S4), yielding 52 more *Pseudomonas* isolates (none had their genome sequenced).

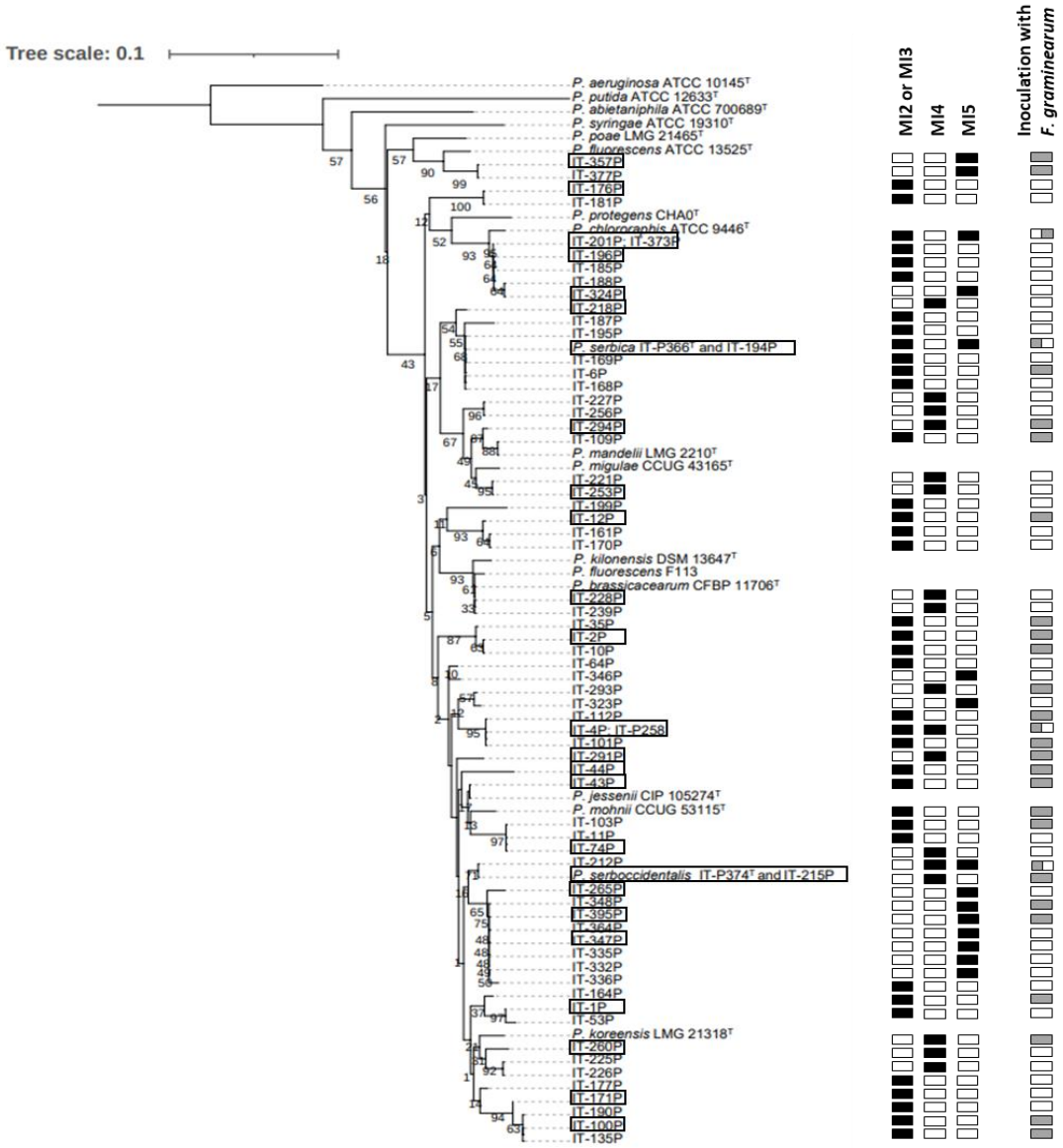


Figure 3. Phylogenetic tree of the 65 *Pseudomonas* with different *rpoD* gene sequences (named IT-x), including 14 *Pseudomonas* type strains (Garrido-Sanz et al., 2016) and *Pseudomonas aeruginosa* ATCC 10145^T, used for tree rooting. The tree was constructed using the SeaView multiplatform (Gouy et al., 2010), with Distance method and 1000 bootstraps, and visualized using iTol (Letunic & Bork, 2021). Strains chosen for whole genome sequencing are framed. For each strain, the soil of origin is indicated (MI2, MI3, MI4 or MI5), and the inoculation status of wheat (gray rectangles when *Fusarium graminearum* Fg1 was used). When two sequenced isolates belonged to the same species (IT-201P and IT-373P, IT-P366 and IT-194P, IT-4P and IT-P258, IT-P374 and IT-215P), but came from different fields, this occurrence is indicated with black rectangles. When one isolate of the same species originated from non-inoculated wheat and the other from inoculated wheat, this is indicated with a rectangle half coloured in gray.

Distribution of whole-genome sequenced *Pseudomonas* strains in soils

The 29 genome-sequenced isolates corresponded to 29 distinct strains. Their affiliation to the *Pseudomonas* genus was confirmed by genome sequencing data. These *Pseudomonas* belonged to the seven subgroups (i.e., *P. fluorescens*, *P. kielensis*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata* and *P. chlororaphis* subgroups) of the *P. fluorescens* group (Figure 4).

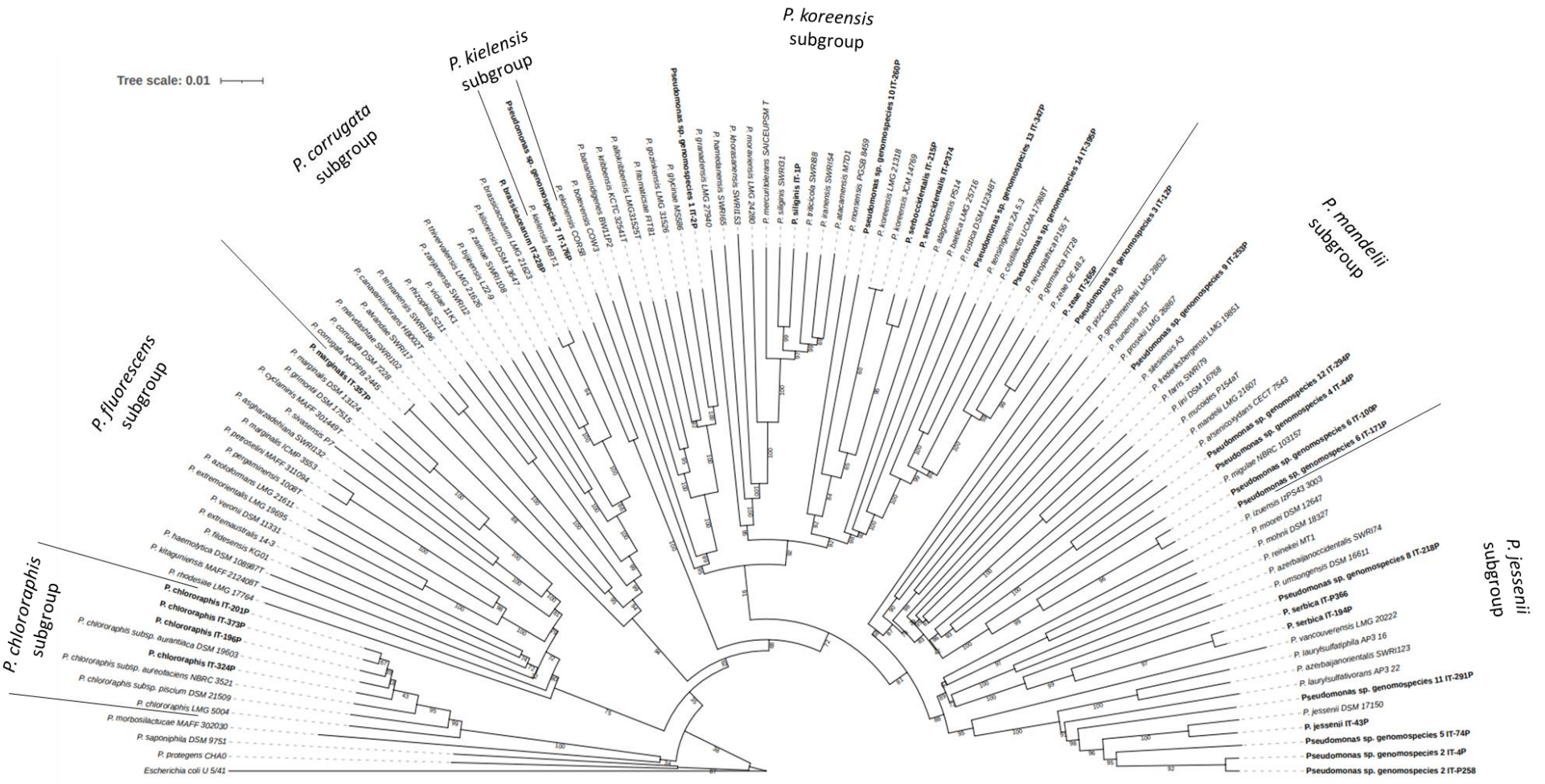


Figure 4. Phylogenetic tree of 29 *Pseudomonas* strains (in bold) chosen for genome sequencing, including *Pseudomonas* type strains (Garrido-Sanz et al., 2016) from the TYGS database and *E. coli* U 5/41^{T}, used for tree rooting. The tree was constructed using TYGS server, inferred with FastME 2.1.6.1 (Lefort et al., 2015) from GBDP distances, calculated from genome sequences. Numbers at the branching points are GBDP pseudo-bootstrap support values from 100 replications. The tree was visualized using iTOL software (Letunic & Bork, 2021).

Digital DNA-DNA hybridization values (dDDH; computed with GGDC 3.0 and formula 2) of the 29 sequenced strains and their closest described *Pseudomonas* type strains (available at the TYGS database) revealed 14 novel genomospecies (hereafter termed *GN-1* to *GN-14*) for 16 of the strains, and their dDDH values were below the threshold of 70% (Table 2), as recommended for species delineation (Chun et al., 2018).

Table 2. The 29 whole-genome sequenced *Pseudomonas* and their distribution in soils (as seen in Figure 4). dDDH values of the 14 novel genomospecies and their closest described species (available at TYGS) are also indicated. dDDH values were calculated using the genome-to-genome distance calculator website service from DSMZ (Meier-Kolthoff & Göker, 2019; Meier-Kolthoff et al., 2022).

Soil	Species name from TYGS	*P. siliginis* SWRI31^T	*P. glycinae* MS586^T	*P. jessenii* DSM 17150^T	*P. piscicola* P50^T	*P. migulae* NBRC 103157^T	*P. kielensis* MBT-1^T	*P. umsongensis* DSM 16611^T	*P. koreensis* LMG 21318^T	*P. silesiensis* A3^T	*P. arsenicoxydans* CECT 7543^T	*P. tensinigenes* ZA 5.3^T	*P. neuropathica* P155^T
MI2	*P. siliginis* IT-1P	*											
	Pseudomonas GN-1 IT-2P		58.2										
	Pseudomonas GN-2 IT-4P			54.6									
	Pseudomonas GN-3 IT-12P				28.9								
	P. jessenii IT-43P												
	Pseudomonas GN-4 IT-44P					41.8							
	Pseudomonas GN-5 IT-74P			52.3									
MI3	*Pseudomonas GN-6* IT-100P					50.4							
	Pseudomonas GN-6 IT-171P					50.9							
	Pseudomonas GN-7 IT-176P						57.4						
	P. serbica IT-194P												
	P. chlororaphis IT-196P												
	P. chlororaphis IT-201P												
MI4	*P. serboccidentalis* IT-215P												
	Pseudomonas GN-8 IT-218P							41.8					
	P. brassicacearum IT-228P												
	Pseudomonas GN-9 IT-253P									33.2			
	Pseudomonas GN-2 IT-P258			53.5									
	Pseudomonas GN-10 IT-260P								43.4				
	P. zeae IT-265P												
	Pseudomonas GN-11 IT-291P			48.4									
	Pseudomonas GN-12 IT-294P										60.9		
MI5	*P. chlororaphis* IT-324P												
	Pseudomonas GN-13 IT-347P											42.8	
	P. marginalis IT-357P												
	P. serbica IT-P366												
	P. chlororaphis IT-373P												
	P. serboccidentalis IT-P374												
	Pseudomonas GN-14 IT-395P												47.3

* For *P. siliginis* IT-1P, dDDH value (GGDC formula 2) with the closest described type strain *P. siliginis* SWRI31^T was 69.3%, but dDDH values for *P. siliginis* IT-1P and *P. siliginis* non-type strain OTU6BANIB1 (GenBank BioSample ID: SAMN29009911) was 80.2, showing that IT-1P and *P. siliginis* OTU6BANIB1 are within the same species.

Different *Pseudomonas* taxa were evidenced in different soils when considering the 29 sequenced *Pseudomonas* strains (Table 2). From the soil MI2, we found five novel genomospecies (*GN-1* to *GN-5*), together with one *P. siliginis* and one *P. jessenii* strain. From the soil MI3, two novel genomospecies were obtained (*GN-6* and *GN-7*), together with one *P. serbica* and two *P. chlororaphis* strains. From the soil MI4, we found six novel genomospecies (*GN-2*, also present in MI2, and *GN-8 to GN-12*), one *P. zeae*, one *P. brassicacearum* and one *P. serboccidentalis* strain. From the soil MI5, two novel genomospecies (*GN-13* and *GN-14*) were evidenced, along with one *P. marginalis*, one *P. serbica*, one *P. serboccidentalis* and two *P. chlororaphis* strains.

Genomic comparison of sequenced *Pseudomonas* strains

In the *P. chlororaphis* subgroup (Figure 4), the four *P. chlororaphis* strains IT-196P, IT-201P (from the soil MI3), IT-324P and IT-373P (from the soil MI5) had a genome size ranging from 6,532 to 7,133 kb, with 6260 to 6872 coding DNA sequences (CDS) and GC content from 62.78% to 63.09% (Table 3). In the *P. koreensis* subgroup, genome sizes of *P. serboccidentalis* IT-215P (from the soil MI4) and IT-P374 (from the soil MI5) were 6,124 kb and 5,997 kb, with 5777 and 5582 CDS and GC contents of 60.29% and 60.36%, respectively. Other strains from the *P. koreensis* subgroup, i.e., *Pseudomonas GN-1* IT-2P, *GN-10* IT-260P, *GN-13* IT-347P, *GN-14* IT-395P, *P. zeae* IT-265P and *P. siliginis* IT-1P had genome size ranging from 5,841 kb to 6,699 kb, 5415 to 6303 CDS, and GC content between 59.10% and 60.51%. The only representative of the *P. kielensis* subgroup, *Pseudomonas GN-7* IT-176P, had genome size of 5,962 kb, 5602 CDS and GC content of 61.20%. In the *P. jessenii* subgroup, *P. serbica* IT-P366 from MI5 soil possessed a larger genome (7,602 kb) than that of *P. serbica* IT-194P from MI3 soil (6,942 kb), due to the presence of a 1,059,298-bp megaplasmid in the former, and possessing 7598 and 6770 CDS, respectively. *P. serbica* IT-P366 had a GC content of 59.55%, while the GC content in *P. serbica* IT-194P was 58.81%. *Pseudomonas* sp. IT-4P from soil MI2 and IT-P258 from soil MI4 (belonging to genomospecies *GN-2* within the *P. jessenii* subgroup) had genome size of 6,312 kb and 6,283 kb, with 5997 and 5915 CDS, and GC content of 59.94% and 59.95%, respectively. Strain IT-4P also contained a 9,290-bp plasmid. *Pseudomonas GN-5* IT-74P, *GN-11* IT-291P and *GN-8* IT-218P, and *P. jessenii* IT-43P had comparable genome sizes (6,304-6,581 kb), CDS (6057-6319) and GC contents (59.61-60.58%). In the *P. mandelii* subgroup, *Pseudomonas GN-6* IT-100P and IT-171P (both from soil MI3) had similar genome sizes (respectively 6,558 and 6,551 kb), CDS (respectively 6313 and 6272) and GC contents (respectively 59.33% and 59.32%). *Pseudomonas GN-3* IT-12P, *GN-9* IT-253P, *GN-12* IT-294P and *GN-4* IT-44P had a genome size between 6,037 and 6,827 kb, with 5799 to 6487 CDS, and a GC content between 58.53% and 61.43%. In the *P. corrugata* subgroup, *P. brassicacearum* IT-228P had a genome size of 6,701 kb, with 6361 CDS and GC content of 60.90%. In the *P. fluorescens* subgroup, *P. marginalis* IT-357P had a genome of 6,611 kb, with 6259 CDS and 61.36% GC content.

When considering soils of origin, genome size was 5,841-6,943 kb for the 13 strains from soil category MI2/MI3 (*P. siliginis* IT-1P, *Pseudomonas GN-1* IT-2P, *Pseudomonas GN-2* IT-4P, *Pseudomonas GN-3* IT-12P, *P. jessenii* IT-43P, *Pseudomonas GN-4* IT-44P, *Pseudomonas GN-5* IT-74P, *Pseudomonas GN-6* strains IT-100P and IT-171P, *Pseudomonas GN-7* IT-176P, *P. serbica* IT-194P, *P. chlororaphis* IT-196P and *P. chlororaphis* IT-201P), 6,023-6,827 kb for the 9 strains from soil MI4 (*P. serboccidentalis* IT-215P, *Pseudomonas GN-8* IT-218P, *P. brassicacearum* IT-228P, *Pseudomonas GN-9* IT-253P, *Pseudomonas GN-2* IT-P258, *Pseudomonas GN-10* IT-260P, *P. zeae* IT-265P, *Pseudomonas GN-11* IT-291P and *Pseudomonas GN-12* IT-294P), and 5,997-7,602 kb for the 7 strains from soil MI5 (*P. chlororaphis* IT-324P, *Pseudomonas GN-13* IT-347P, *P. marginalis* IT-

357P, *P. serbica* IT-P366, *P. chlororaphis* IT-373P, *P. serboccidentalis* IT-P374 and *Pseudomonas GN-14* IT-395P) (Table 3). GC content was 59.32-63.09% for the 13 strains from soils MI2/MI3, 58.53-60.90% for the 9 strains from soil MI4 and 59.19-62.99% for the 7 strains from soil MI5. In summary, genome size and GC content of the 29 sequenced strains depended on the species or subgroup, regardless of the soil of origin.

Table 3. Genomic features of sequenced *Pseudomonas,* retrieved from TYGS.

Soil	Species name from TYGS	Isolate name	Genome size (bp)	Plasmid	GC content (%)	No. contigs	Coding DNA sequences (CDS)
MI2	*P. siliginis*	IT-1P	5,841,413	-	60.07	37	5415
	Pseudomonas GN-1	IT-2P	6,478,735	-	60.51	49	6041
	Pseudomonas GN-2	IT-4P	6,312,045	+	59.94	111	5997
	Pseudomonas GN-3	IT-12P	6,341,720	-	61.43	28	6001
	P. jessenii	IT-43P	6,413,346	-	59.66	75	6124
	Pseudomonas GN-4	IT-44P	6,569,010	-	59.57	101	6263
	Pseudomonas GN-5	IT-74P	6,304,484	-	59.87	93	6057
MI3	*Pseudomonas GN-6*	IT-100P	6,558,007	-	59.33	59	6313
	Pseudomonas GN-6	IT-171P	6,551,484	-	59.32	60	6272
	Pseudomonas GN-7	IT-176P	5,962,660	-	61.20	123	5602
	P. serbica	IT-194P	6,942,565	-	59.81	94	6770
	P. chlororaphis	IT-196P	6,635,492	-	63.09	29	6284
	P. chlororaphis	IT-201P	6,532,202	-	62.84	23	6260
MI4	*P. serboccidentalis*	IT-215P	6,124,801	-	60.29	67	5777
	Pseudomonas GN-8	IT-218P	6,581,279	-	60.58	96	6319
	P. brassicacearum	IT-228P	6,701,129	-	60.90	74	6361
	Pseudomonas GN-9	IT-253P	6,037,596	-	58.53	84	5799
	Pseudomonas GN-2	IT-P258	6,283,203	-	59.95	109	5915
	Pseudomonas GN-10	IT-260P	6,023,190	-	60.33	44	5566
	P. zeae	IT-265P	6,699,764	-	59.10	126	6303
	Pseudomonas GN-11	IT-291P	6,322,035	-	59.61	103	6098
	Pseudomonas GN-12	IT-294P	6,827,290	-	58.98	59	6487
MI5	*P. chlororaphis*	IT-324P	7,133,109	-	62.78	56	6872
	Pseudomonas GN-13	IT-347P	6,284,985	-	59.46	60	5743
	P. marginalis	IT-357P	6,611,256	-	61.36	47	6259
	P. serbica	IT-P366	7,601,897	+	59.55	93	7598
	P. chlororaphis	IT-373P	6,801,379	-	62.99	16	6486
	P. serboccidentalis	IT-P374	5,997,322	-	60.36	39	5582
	Pseudomonas GN-14	IT-395P	6,472,514	-	59.19	61	5965

Presence of genes involved in biocontrol or plant growth promotion

BLAST revealed the presence of genes for biosynthesis of antimicrobial compounds in most of the 29 *Pseudomonas* strains sequenced (Table 4). In the *P. chlororaphis* subgroup, the four *P. chlororaphis* strains, i.e., IT-196P, IT-201P (from the soil MI3), IT-324P and IT-373P (from the soil MI5), harbored genes involved in production of phenazine, pyrrolnitrin, HCN, pyoverdine, ethylene, auxin, 2,3-butanediol conversion to acetoin, acetoin catabolism, phosphate solubilization, denitrification and *aprA* genes for production of alkaline metalloproteinase. All *P. chlororaphis* strains harbored genes for HPR production but strain IT-201P lacked *darC*. Additionally, *P. chlororaphis* IT-324P had the *fitD* insect-toxin gene, involved in control of insect pests. The highest number of putative biosynthetic gene clusters (BGCs) was found in *P. chlororaphis* strains, up to 16 (Table S2). antiSMASH revealed the presence of a complete region for massetolide A in strains IT-196P, IT-201P and IT-373P (Table S3). An operon for type VI secretion system was detected in all *P. chlororaphis* strains, and for type III secretion system in strain IT-324P. All *P. chlororaphis* strains harbored genes for chitinases and betaglucanases (except IT-201P) (Figure S5), and genes of the AA10 family (which includes lytic polysaccharide monooxygenases that potentially target chitin) (Figure S6), but none of them harbored genes for cellulases and mannanases.

In the *P. koreensis* subgroup, *P. serboccidentalis* strains IT-215P (from the soil MI4) and IT-P374 (from the soil MI5) harbored genes for production of HCN, pyoverdine, alkaline metalloproteinase and *gcd*/*gad* genes for phosphate solubilization. antiSMASH revealed a type VI secretion system operon in both *P. serboccidentalis* strains (Table S3). Annotation of CAZymes showed that genomes of these two *P. serboccidentalis* strains contained genes involved in production of chitinases and genes of the AA10 family (Figure S5). Strains *Pseudomonas GN-1* IT-2P (from MI2), *GN-10* IT-260P (from MI4), *GN-13* IT-347P (from MI5), *GN-14* IT-395P (from MI5), *P. zeae* IT-265P (from MI4) and *P. siliginis* IT-1P (from MI2) harbored *gcd*/*gad* genes for phosphate solubilization. These strains contained genes for HCN, pyoverdine and ethylene production (except *P. siliginis* IT-1P, which harbored only genes for HCN and ethylene production). Gene *aprA* for production of alkaline metalloproteinase was harbored by *Pseudomonas GN-1* IT-2P, *GN-13* IT-347P, *GN-14* IT-395P, *P. zeae* IT-265P and *P. siliginis* IT-1P. These strains also contained an operon for a type VI secretion system, while *P. zeae* IT-265P also contained genes for a type III secretion system. These six strains also had the potential of producing chitinases and lytic polysaccharide monooxygenases, and some of them (IT-1P, IT-2P and IT-347P) also had the potential of producing betaglucanases (Figure S5 and Figure S6).

In the *P. kielensis* subgroup, *Pseudomonas GN-7* IT-176P (from MI3) possessed genes for 2,3-butanediol conversion to acetoin, for acetoin catabolism and *gcd* gene for phosphate solubilization. It also harbored an operon for a type VI secretion system (Table S3). Additionally, this strain could potentially produce chitinases and betaglucanases, as shown by the annotation of CAZymes (Figure S5).

Table 4. Distribution of genes involved in biocontrol and plant-growth promotion in the *Pseudomonas* strains. Presence of the property (the whole gene cluster) is marked with +, and when for certain property there are several possible pathways to achieve a function, names of the genes found in the genome are indicated. Genes were found with DIAMOND blastp (v.2.0.8.146; Buchfink et al., 2015), using the options --query-cover 80 --id 70 (query coverage >80%; amino acid identity >70%), if not specified otherwise. Origin of each strain is indicated based on soil (MI2, MI3, MI4 or MI5) and inoculation status of wheat used for isolation (i for inoculation with *Fusarium graminearum* Fg1 and c for non-inoculated wheat).

Soil	Fg1 inoculation	Isolate name	Phenazine production	HPR production	DAPG production	Pyrrolnitrin production	HCN production	Pyoverdine production	Ethylene production	ACC deaminase	Auxin biosynthesis	2,3-butanediol conversion to acetoin	Acetoin catabolism	Phosphate solubilization	Denitrification	Alkaline metalloproteinase production	FitD production	Number of phytobenefical functions
MI2	i	*P. siliginis* IT-1P					+		+					*gcd, gad*		+		4
	i	*Pseudomonas GN-1* IT-2P					+	+	+					*gcd, gad*		+		5
	i	*Pseudomonas GN-2* IT-4P						+	+		*iaaMH**			*gcd*				4
	i	*Pseudomonas GN-3* IT-12P								+	*iaaMH**				*nirS*			3
	i	*P. jessenii* IT-43P						+	+		*iaaMH**			*gcd*				4
	i	*Pseudomonas GN-4* IT-44P						+	+	+	*iaaMH**				*nirS*	+		6
	c	*Pseudomonas GN-5* IT-74P						+	+					*gcd*		+		4
MI3	i	*Pseudomonas GN-6* IT-100P						+		+					*nirS*	+		4
	c	*Pseudomonas GN-6* IT-171P						+		+					*nirS*	+		4
	c	*Pseudomonas GN-7* IT-176P										*adh*	+	*gcd*				3
	c	*P. serbica* IT-194P						+	+		*iaaMH**							3

	c	*P. chlororaphis* IT-196P	+	+		+	+	+	+		*iaaMH*	*bdhA, adh*	+	*gcd, gad*	*nirK*	+		12
	c	*P. chlororaphis* IT-201P	+			+	+	+	+		*iaaMH*	*bdhA, adh*	+	*gcd, gad*	*nirK*	+		11
MI4	c	*P. serboccidentalis* IT-215P					+	+						*gcd, gad*		+		4
	c	*Pseudomonas GN-8* IT-218P						+	+					*gad*				3
	c	*P. brassicacearum* IT-228P			+		+			+	*iaaMH**	*adh*	+		*nirS*	+		8
	c	*Pseudomonas GN-9* IT-253P																0
	c	*Pseudomonas GN-2* IT-P258							+					*gcd*				2
	i	*Pseudomonas GN-10* IT-260P					+	+	+					*gcd, gad*				4
	i	*P. zeae* IT-265P					+	+	+					*gcd, gad*		+		5
	i	*Pseudomonas GN-11* IT-291P										*adh*		*gcd*				2
	i	*Pseudomonas GN-12* IT-294P												*gad*	*nirS*	+		3
MI5	c	*P. chlororaphis* IT-324P	+	+		+	+	+	+		*iaaMH*	*bdhA, adh*	+	*gcd, gad*	*nirK*	+	+	13
	c	*Pseudomonas GN-13* IT-347P					+	+	+					*gcd, gad*		+		5
	i	*P. marginalis* IT-357P								+		*adh*	+	*gad*	*nirS*	+		6
	i	*P. serbica* IT-P366							+		*iaaMH**							2
	i	*P. chlororaphis* IT-373P	+	+		+	+	+	+		*iaaMH*	*bdhA, adh*	+	*gcd, gad*	*nirK*	+		12
	i	*P. serboccidentalis* IT-P374					+	+						*gcd, gad*		+		4
	i	*Pseudomonas GN-14* IT-395P					+	+	+					*gcd, gad*		+		5

Genes (and functions) that were searched for in the 29 *Pseudomonas* isolates, and were not found: *pltABCDEFGLM* (production of pyoluteorin), *pchABCDEF* (production of pyochelin), *pmsABCE* (production of pseudomonine), *iacABCDEFGHI* (auxin catabolism), *budB/ilvNB/alsS*, *budA/alsD* (acetoin biosynthesis), *budC/ydjL* (2,3-butanediol biosynthesis) and *nifHDK* (nitrogen fixation).
* *iaaH* found with only 30-40 % identity

In the *P. jessenii* subgroup, *P. serbica* IT-P366 (from MI5 soil) and IT-194P (from MI3 soil) possessed genes involved in the modulation of plant hormonal levels, i.e., for auxin biosynthesis and ethylene production (Table 4). Moreover, *P. serbica* IT-194P harbored *pvdL* gene for pyoverdine production. Both strains host an operon for type VI secretion system, and the megaplasmid of IT-P366 displays an operon for the synthesis of a type IV secretion system (Dot/Icm family; Table S3). Both strains also contained genes for chitinases and betaglucanases production (Figure S5), and genes of the AA10 family (Figure S6), but only IT-194P possessed genes for cellulase production. *Pseudomonas GN-2* strains IT-4P (from soil MI2) and IT-P258 (from soil MI4) harbored *gcd* (phosphate solubilization) and *efe* (ethylene production), and strain IT-4P has the potential of producing pyoverdine and auxin. These two strains contained an operon involved in synthesis of a type VI secretion system, as well as genes of the AA10 family, and IT-P258 contained chitinases genes. *Pseudomonas GN-5* IT-74P (from MI2), *GN-11* IT-291P (from MI4) and *GN-8* IT-218P (from MI4), and *P. jessenii* IT-43P (from MI2) harbored *gcd* and all four but IT-291P possessed genes for pyoverdine and ethylene production. Moreover, IT-43P contained genes for auxin biosynthesis, IT-74P displayed genes for alkaline metalloproteinase production and IT-291P contained genes for 2,3-butanediol conversion to acetoin. Strains *GN-5* IT-74P, *GN-11* IT-291P and *GN-8* IT-218P also contained an operon involved in synthesis of a type VI secretion system. Strains IT-218P and IT-291P had genes for cellulases, IT-74P and IT-218P had genes for chitinases, IT-43P and IT-218P had genes for betaglucanases, while all but IT-74P had genes of the AA10 family.

In the *P. mandelii* subgroup, *Pseudomonas GN-6* IT-100P and IT-171P (both from soil MI3) have the potential of producing pyoverdine, ACC deaminase, alkaline metalloproteinase and for denitrification (Table 4). Both displayed an operon for type III and type VI secretion systems (Table S3), genes for chitinases and betaglucanases (Figure S5), as well as genes of the AA10 family (Figure S6). *Pseudomonas GN-3* IT-12P (MI2 soil) had *nirS* and genes for ACC deaminase and auxin production. *Pseudomonas GN-4* strain IT-44P, also from the MI2 soil, harbored the same three genes and also genes for pyoverdine, ethylene and alkaline metalloproteinase production. *Pseudomonas GN-12* IT-294P (soil MI4) had genes for P solubilization, denitrification and alkaline metalloproteinase production. *Pseudomonas GN-9* IT-253P (from MI4) harbored none of the genes investigated. antiSMASH revealed a type VI secretion system operon in IT-44P, IT-294P and IT-253P, and a type III secretion system operon in IT-253P. Inspection of CAZymes showed that the last four strains harbored genes for betaglucanases, all but IT-253P harbored genes of the AA10 family and chitinases, and only IT-44P and IT-253P harbored cellulases genes.

In the *P. corrugata* subgroup, *P. brassicacearum* IT-228P (from soil MI4) was the only one harboring genes for DAPG production, besides genes for HCN, ACC deaminase, auxin and alkaline metalloproteinase production, and genes for 2,3-butanediol conversion to acetoin, acetoin catabolism and denitrification. This strain also had operons for type III and type VI secretion systems (Table S3), and the potential of producing chitinases (CAZyme annotation).

Finally, in the *P. fluorescens* subgroup, *P. marginalis* IT-357P (from soil MI5) had genes for ethylene and alkaline metalloproteinase production, phosphate solubilization, denitrification, and genes for 2,3-butanediol conversion to acetoin and acetoin catabolism. It also contained operons for type III and type VI secretion systems (Table S3), and genes for production of chitinases, cellulases and genes of the AA10 family (Figure S5 and S6).

Altogether, HCN genes were the most common (in 13 strains from all soils), followed by those for pyrrolnitrin and phenazine (each present in four strains from soils MI3 or MI5), HPR (three strains from soils MI3 or MI5) and DAPG (one MI4 strain). None had pyoluteorin genes. Many strains (18 of 29, from all soils) also possessed an *aprA* protease gene and one MI5 strain

the *fitD* insect-toxin gene. The pyoverdine gene *pvdL* was found in 19 strains (from all soils), and all genomes shared partial homologies with siderophore BGCs known from *Pseudomonas* (pyoverdine) as well as non-*Pseudomonas* bacteria. Genes for secondary siderophores pyochelin and pseudomonine were not detected. Most of the strains (from all soils) had the potential for interfering with plant hormonal levels, i.e., 18 harbored *efe*, 6 possessed *acdS*, 11 displayed *iaaM* and *iaaH* (though 7 strains had only 30-40% identity with the query *iaaH* from *Pseudomonas* JV395A), but none had auxin catabolism genes. Eight strains (from all soils) had *adh* and four of them had also *bdhA*, but none of them harbored genes for acetoin or 2,3-butanediol synthesis. In addition, seven strains (from all soils) had *aco* genes for acetoin catabolism. Many strains (from all soils) displayed genes influencing plant nutritional status, via phosphate solubilization (*gcd* and/or *gad* in 21 strains) and denitrification (*nirK*/*nirS* in 11 strains), while *nifHDK* were not found.

In summary, the genes involved in phytobeneficial functions were spread quite evenly among *Pseudomonas* strains regardless of the experimental conditions (field of origin, suppressiveness status, previous manure application; Table 4). Yet, the biosynthetic genes for antimicrobial compounds phenazine(s), HPR and pyrrolnitrin were restricted to four *P. chlororaphis* strains from MI3 (manure used; fungistatic and suppressive) or MI5 (no manure; non-fungistatic and suppressive). *Pseudomonas* strains from all three types of soils possessed from 0 to 13 genes (in *P. chlororaphis*) coding for phytobeneficial functions, which were evenly distributed, regardless of the soil of origin (Table 4).

Presence of biocontrol and plant growth promotion functions

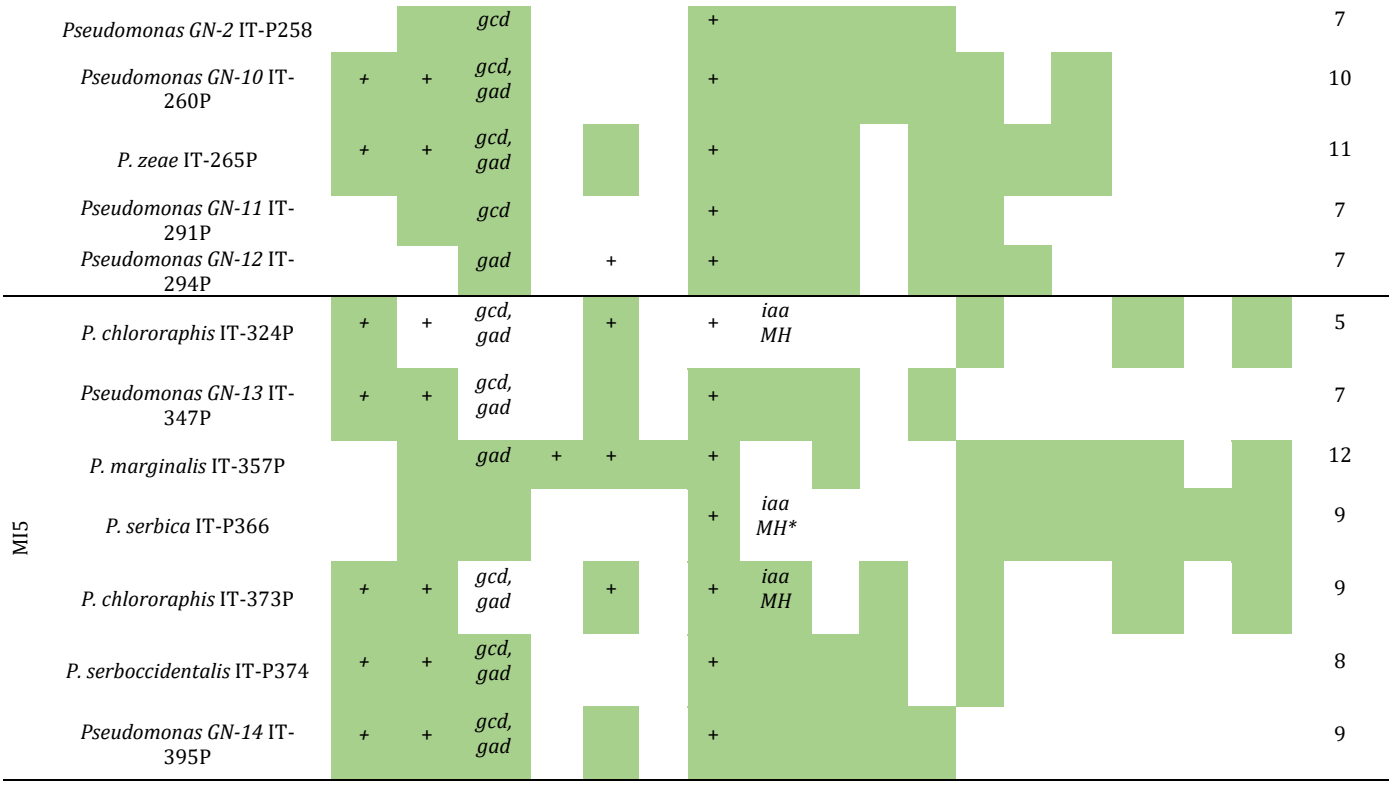
The 29 *Pseudomonas* were tested *in vitro* for traits contributing to biocontrol or plant growth promotion. In the *P. chlororaphis* subgroup, the MI3 isolates IT-196P and IT-201P and MI5 isolates IT-324P and IT-373P had the ability to produce HCN, siderophore, chitinase (except IT-324P from soil MI5), proteases (except IT-196P from soil MI3), but not cellulases (Table 5). They also produced indole-3-acetic acid and indole-3-carboxylic acid (except IT-324P), trans-zeatin, isopentenyl adenosine and kynurenic acid, whereas trans-zeatin riboside and 6-benzylaminopurine were produced only by strain IT-201P from soil MI3, and none solubilized phosphates.

In the *P. koreensis* subgroup, all eight strains produced HCN, siderophores, chitinases, indole-3-acetic acid and indole-3-lactic acid. All but *P. serboccidentalis* IT-215P (soil MI4) and *Pseudomonas GN-13* IT-347P (soil MI5) solubilized inorganic sources of P and produced indole-3-propionic acid. Proteases were produced by five strains (IT-1P from soil MI2, IT-215P and IT-265P from soil MI4, IT-347P and IT-395P from soil MI5), while cellulases were produced only by *P. siliginis* IT-1P (from soil MI2). Indole-3-carboxylic acid was produced by four strains (IT-215P and IT-260P from soil MI4, IT-P374 and IT-395P from soil MI5), trans-zeatin by six strains (IT-1P and IT-2P from soil MI2, IT-215P, IT-260P and IT-265P from soil MI4, and IT-P374 from soil MI5), trans-zeatin riboside by three strains (IT-1P and IT-2P from soil MI2, IT-265P from soil MI4), 6-benzylaminopurine by four strains (IT-1P from soil MI2, IT-215P, IT-260P and IT-265P from soil MI4), isopentenyl adenosine by three strains (IT-1P and IT-2P from soil MI2, IT-215P from soil MI4), and kynurenic acid only by two strains (IT-2P from soil MI2 and IT-215P from soil MI4).

In the *P. kielensis* subgroup, *Pseudomonas GN-7* IT-176P (soil MI3) was able to produce siderophores and proteases. It solubilized phosphates and produced trans-zeatin, trans-zeatin riboside, 6-benzylaminopurine, isopentenyl adenosine, abscisic acid and kynurenic acid.

Table 5. Correspondence between gene presence and *in vitro* activities involved in plant-growth promotion and biocontrol in 29 *Pseudomonas,* according to the soil of origin. Activity is marked with a green colour. Gene corresponding to a given activity *in vitro* (when found in the genomes) is indicated with + (the whole gene cluster), and when for certain property there are several possible pathways to achieve a function, names of the genes found in the genome are indicated. Cellulases and chitinases were predicted using dbCAN2 (v.3; Zhang et al., 2018) and compared with the CAZy database using HMMER (v.3.3; Eddy, 2011). Prediction of function and substrate specificity of CAZyme families or subfamilies was performed based on a review of activities assigned to CAZymes with known structures (characterized enzymes) in the CAZy database (http://www.cazy.org) (Lombard et al., 2014) and manually curated, as previously described (López-Mondéjar et al., 2022).

Soil	Strain name	HCN	Siderophores	Phosphate solubilization	ACC deaminase	Protease	Cellulase	Chitinase	Indole-3-acetic acid	Indole-3-lactic acid	Indole-3-carboxylic acid	Indole-3-propionic acid	Trans-zeatin	Trans-zeatin riboside	6-Benzylaminopurine	Isopentenyl adenosine	Abscisic acid	Kynurenic acid	Number of phytobenefical functions
MI2	*P. siliginis* IT-1P	+		*gcd, gad*		+		+											13
	Pseudomonas GN-1 IT-2P	+	+	*gcd, gad*		+		+											10
	Pseudomonas GN-2 IT-4P		+	*gcd*				+	*iaa MH**										7
	Pseudomonas GN-3 IT-12P				+			+	*iaa MH**										12
	P. jessenii IT-43P		+	*gcd*				+	*iaa MH**										7
	Pseudomonas GN-4 IT-44P		+		+	+		+	*iaa MH**										12
	Pseudomonas GN-5 IT-74P		+	*gcd*		+		+											8
MI3	*Pseudomonas GN-6* IT-100P		+		+	+		+											7
	Pseudomonas GN-6 IT-171P		+		+	+		+											9
	Pseudomonas GN-7 IT-176P			*gcd*				+											9
	P. serbica IT-194P		+					+	*iaa MH**										8
	P. chlororaphis IT-196P	+	+	*gcd, gad*		+		+	*iaa MH*										8
	P. chlororaphis IT-201P	+	+	*gcd, gad*		+		+	*iaa MH*										11
MI4	*P. serboccidentalis* IT-215P	+	+	*gcd, gad*				+											12
	Pseudomonas GN-8 IT-218P		+	*gad*				+											7
	P. brassicacearum IT-228P	+			+	+		+	*iaa MH**										11
	Pseudomonas GN-9 IT-253P							+											6



* *iaaH* found with only 30-40 % identity; none of the isolates produced indole-3-pyruvic acid, indole-3-butyric acid, tryptophol, kinetin, gibberellin A1 or gibberellic acid.

In the *P. jessenii* subgroup, *P. serbica* IT-P366 (from MI5) and IT-194P (from MI3) were able to solubilize phosphates and to produce siderophores, chitinase, and phytohormones (trans-zeatin, 6-benzylaminopurine, isopentenyl adenosine, abscisic acid and kynurenic acid), while only strain IT-P366 could produce trans-zeatin riboside. *Pseudomonas GN-2* IT-4P (from MI2) and IT-P258 (from MI4) displayed P solubilization, production of siderophores, chitinase, indole-3-acetic acid, indole-3-lactic acid and indole-3-propionic acid, but only IT-P258 produced indole-3-carboxylic acid and IT-4P trans-zeatin. *Pseudomonas GN-5* IT-74P (soil MI2), *GN-11* IT-291P (soil MI4) and *GN-8* IT-218P (soil MI4), and *P. jessenii* IT-43P exhibited production of siderophores, chitinase and trans-zeatin, and P solubilization. Indole-3-acetic acid, indole-3-lactic acid and indole-3-propionic acid were produced by all strains except IT-218P. Contrarily, indole-3-carboxylic acid, trans-zeatin riboside and kynurenic acid were produced only by IT-218P and proteases by IT-74P.

In the *P. mandelii* subgroup, *Pseudomonas* sp. IT-100P and IT-171P (*GN-6*, both from soil MI3) produced siderophores, ACC deaminase, protease, indole-3-acetic acid, indole-3-lactic acid and indole-3-propionic acid, and they solubilized phosphate. Strain IT-171P also produced chitinase and indole-3-carboxylic acid. *Pseudomonas GN-3* IT-12P (soil MI2), *GN-9* IT-253P (soil MI4), *GN-12* IT-294P (soil MI4) and *GN-4* IT-44P (soil MI2) had in common production of chitinase, indole-3-propionic acid, trans-zeatin and trans-zeatin riboside, as well as phosphate solubilization. Siderophores were produced by all strains (except IT-294P), and indole-3-acetic acid and indole-3-lactic acid by all strains (but IT-253P). Additionally, strains IT-12P and IT-44P produced ACC deaminase, 6-benzylaminopurine and isopentenyl adenosine. Protease was produced only by IT-44P and indole-3-carboxylic acid only by IT-12P.

In the *P. corrugata* subgroup, *P. brassicacearum* IT-228P produced HCN, siderophores, ACC deaminase, protease, and several phytohormones. They included indole-3-acetic acid,

indole-3-lactic acid, indole-3-propionic acid, trans-zeatin, trans-zeatin riboside, 6-benzylaminopurine and isopentenyl adenosine.

In the *P. fluorescens* subgroup, strain *P. marginalis* IT-357P solubilized phosphate and produced siderophores, ACC deaminase, protease, cellulase, chitinase. It also produced the phytohormones indole-3-lactic acid, trans-zeatin, trans-zeatin riboside, 6-benzylaminopurine, isopentenyl adenosine and kynurenic acid.

Correspondence between gene presence and *in vitro* activity

Out of the activities tested *in vitro*, production of HCN and ACC-deaminase activity corresponded well to the presence of the corresponding genes, and was recorded in *Pseudomonas* strains from all three soil categories (Table 5). All but *Pseudomonas GN-12* IT-294P from MI4 and *P. chlororaphis* IT-324P from MI5 produced siderophores *in vitro*. *In vitro* phosphate solubilization activity corresponded to the presence of *gcd* and/or *gad* genes in 15 of 22 strains but not in 7 other strains, indicating other P solubilization mechanisms. In addition, 6 strains possessed both *gcd* and *gad* but did not solubilize phosphate under the conditions tested. Similarly, the presence of *aprA* matched with the *in vitro* proteolytic activities in 10 strains, while in 5 strains activity was present but not *aprA*, suggesting the involvement of other protease genes. In 3 strains, *aprA* gene was found, but without activity. Most strains produced chitinases, in accordance with presence of chitinase genes (Figure S5). Cellulase activity was found only in *P. siliginis* IT-1P (which displays GH3 family genes acting on cellobiose) and *P. marginalis* IT-357P (with genes for cellulose degradation) (Figure S5 and S6). Most strains produced indole-3-acetic acid, but only eleven of them harbored *iaaMH* genes for auxin synthesis, and none of the isolates harbored *ipdC* or *ppdC* genes. Indole-3-pyruvic acid, indole-3-butyric acid, tryptophol, kinetin, gibberellin A1 or gibberellic acid were not produced by any of the strains, while all the other phytohormones tested were produced by strains from all soils. In conclusion, all phenotypic traits tested were found in isolates from all three soil categories, and strains with higher (12 or 13) phytobeneficial functions were isolated from all three soil categories.

Correspondence between gene presence and *in vitro* activity matched in the case of HCN and ACC-deaminase productions, where all the gene(s) involved in the pathway are known. However, for production of siderophores, protease, cellulase and chitinase, P solubilization, and indole-3-acetic acid production, gene presence did not coincide with activity in all strains, indicating the involvement of other genes (in the case when there was activity, without finding the gene(s) that we searched for) or non-expression of the gene (in the case when the gene is present, but without the activity).

Inhibitory effect of *Pseudomonas* volatile organic compounds on fungal growth and inhibitory effect of *Pseudomonas* exudates on sporulation of *Fusarium graminearum*

Growth inhibition of *F. graminearum* Fg1 by VOCs produced by *Pseudomonas* strains was >20% with *P. marginalis* IT-357P (31.6%) and *Pseudomonas GN-14* IT-395P (30.6%) from soil MI5 and *P. serboccidentalis* IT-215P (21.5%) from soil MI4. Inhibition was below 20% for *P. brassicacearum* IT-P228 (15.4%) and *Pseudomonas GN-9* IT-253P (14.0%) from soil MI4, and *Pseudomonas GN-1* IT-2P (14%) from soil MI2 and *P. chlororaphis* IT-196P (18.9%) from soil MI3.

Spore germination inhibition was only observed with the two strains from MI5 soil (13.5% with *P. marginalis* IT-357P and 18.1% with *Pseudomonas GN-14* IT-395P). In summary, from the 29 *Pseudomonas* tested, seven of them (originating from soil categories MI2/MI3, MI4

and MI5) were able to inhibit growth of *F. graminearum* Fg1 by production of VOCs, and two of them (both from MI5 soil) were able to inhibit fungal spore germination.

DISCUSSION

Comparisons of soils suppressive vs. conducive to *Fusarium* diseases have revealed differences in the occurrence or prevalence of various taxa (Cha et al., 2016; Siegel-Hertz et al., 2018; Ossowicki et al., 2020; Lv et al., 2023), and microorganisms associated with suppressive conditions are likely to contribute to plant protection. In this context, we focused on *Pseudomonas*, one of the key taxa thought to play a role in disease suppressiveness. Their genetic characteristics enable them to colonize different soils, including disease-suppressive soils (Weller et al., 2007; Kyselková & Moënne-Loccoz, 2012; Santoyo et al., 2012), and they exhibit a wide range of plant-growth promoting and biocontrol properties, such as producing antifungal compounds, competing with pathogens and triggering ISR in plants (Kloepper et al., 1980; Sneh et al., 1984; Weller et al., 2007; Almario et al., 2013a; Almario et al., 2013b; Vacheron et al., 2016; Legrand et al., 2019; Shen et al., 2022). Shen et al. (2022) suggested that *Pseudomonas* populations might be stimulated in suppressive soils, due to the pathogen pressure and dynamic interactions with the other microbial populations. Additionally, *Pseudomonas* with biocontrol properties were already isolated from suppressive soils, including *Pseudomonas* sp. Q2-87 (*P. corrugata* subgroup; Weller et al., 2007), isolated from wheat in take-all decline soils but that protects tomato from *F. oxysporum* f. sp*. radicis-lycopersici* and *Pseudomonas* sp. C7 (*P. corrugata* subgroup; Lemanceau & Alabouvette, 1991) isolated from soil suppressive to Fusarium wilt of tomato.

In the present study, we used rhizospheres of wheat plants (inoculated or not with *F. graminearum* Fg1), grown in soils with different behaviors related to fungistasis and suppressiveness to *F. graminearum* Fg1 disease, and with different history of manuring. Soils MI2 and MI3 were fungistatic and suppressive, and both previously received manure treatment, soil MI4 was non-fungistatic and non-suppressive, without manure application, whereas soil MI5 was non-fungistatic but is suppressive, without previous manure treatment (Todorović et al., submitted; Chapter 2). Organic amendments, such as animal manure, are thought to improve soil health by stimulating plant-beneficial microbiota (Mousa & Raizada, 2016) and enhancing microbial diversity (Shu et al., 2022), which was perhaps instrumental in conferring fungistasis. Accordingly, soils that received manure amendments were more fungistatic than non-amended soils towards *F. graminearum* (Legrand et al., 2019), and 17 of 18 composts (made from different mixtures of manure, domestic biowaste and green waste) conferred protection from Fusarium wilt caused by *F. oxysporum* f. sp. *lini* (Termorshuizen et al., 2006). Bio-organic fertilizer reshaped the soil microbiome and particularly stimulated indigenous *Pseudomonas* community, providing suppression to Fusarium wilt disease of banana (Tao et al., 2020). The non-amended soil MI5 is non-fungistatic but suppressive, probably resulting from ISR-triggering or direct pathogen inhibition of the rhizosphere microbiota (including *Pseudomonas*) on roots (Tamietti & Matta, 1984; Tamietti & Alabouvette, 1986; Bakker et al., 2007; Kyselková & Moënne-Loccoz, 2012).

Here, the *rpoD* primers of Manriquez (2021) were used for the first time for metabarcoding analysis of *Pseudomonas* populations in suppressive soils. Comparison of soils MI2/MI3, MI4 and MI5 did not evidence any significant difference in α-diversity between the three soil categories (Figure 1), except that Simpson index (which measures both richness and relative abundance; Hagerty et al., 2020) was significantly lower for soil MI4 (non-fungistatic and non-suppressive) compared with the others. A higher Simpson index was also evidenced in soils suppressive to wilt disease of banana mediated by *F. oxysporum* f. sp. *cubense* (compared with

conducive soils), but this was at the scale of the total bacterial community (Nisrina et al., 2021). *Pseudomonas* taxonomic composition differed between all three soil conditions (Figure 2). This is reminiscent of denaturing gradient gel electrophoresis results showing differences in DAPG$^+$ *Pseudomonas* (sub)populations in soils suppressive or conducive to Thielaviopsis black root rot of tobacco (Frapolli et al., 2010).

In the current study, we isolated 406 putative fluorescent *Pseudomonas* using soils MI2, MI3, MI4 and MI5, and the analysis resulted in the identification of 65 *Pseudomonas* strains with unique sequences, from which 29 *Pseudomonas* were selected for whole genome sequencing (13 from soils MI2 and MI3, 9 from soil MI4 and 7 from soil MI5; 16 of them came from wheat inoculated with *F. graminearum* Fg1 and 13 from non-inoculated wheat; Figure 3). Genome-sequenced *Pseudomonas* from this study belonged to subgroups *P. fluorescens*, *P. kielensis*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata* and *P. chlororaphis* of the *P. fluorescens* group. The distribution of these 29 sequenced *Pseudomonas* taxa was rather soil-specific, as found for 6 of 7 species in soil MI2, 2 of 4 species in soil MI3, 7 of 9 species in soil MI4, and 3 of 6 species in soil MI5. This is largely in line with our *rpoD* metabarcoding data, but contrasts with *phlD*-based DGGE findings in another type of suppressive soils (Frapolli et al., 2010). Whole-genome sequencing of the 29 *Pseudomonas* identified as many as 14 novel genomospecies (one of them, *GN-2*, found both in soils MI2 and MI4). This deserves further research to formally describe these 14 potentially-new species and assess their significance in suppressive soils, in Serbia and elsewhere.

Genome analysis of the 29 strains evidenced taxonomic particularities (Table 4). Notably, the four *P. chlororaphis* strains, which originated from MI3 and MI5 (both suppressive to *F. graminearum* disease), harbored as many as 11 to 13 genes involved in biocontrol or plant-growth promotion. This may reflect the taxonomy rather than the soil origin of the strains, as the ability of *P. chlororaphis* to produce compounds with antimicrobial activity (Arseneault & Filion, 2016) and protect plant is well documented (Raio & Puopolo, 2021). *P. brassicacearum* IT-228P (from the non-suppressive soil MI4) was the only isolate able to produce DAPG, a prominent biocontrol metabolite in several types of suppressive soils (Weller et al., 2007; Frapolli et al., 2010). Characterization of activities *in vitro* revealed a wide distribution among the 29 strains, with again taxonomic particularities (with up to 13 phytobeneficial functions per strain). Nevertheless, when comparing the soils of origin, there was a rather even distribution of plant-growth promoting and biocontrol properties (both genetic and phenotypic) among the strains, regardless of the experimental conditions (e.g., soil suppressiveness/fungistasis status and inoculation status) (Table 4 and Table 5). It is known that *Pseudomonas* strains with biocontrol potential can also be found in conducive soils (Ramette et al., 2006; Frapolli et al., 2010). Therefore, it will be important to assess whether suppressiveness is associated with (i) a particular relative abundance of *Pseudomonas* genotypes, as they may differ in phytoprotection capacity (Ramette et al., 2006; Weller, 2007), or (iii) particular levels of expression of biocontrol genes (Ramette et al., 2003; Almario et al., 2013b).

In conclusion, rather than pointing ecological and genomic particularities of fluorescent *Pseudomonas* from suppressive soil that contrast with counterparts in non-suppressive soils (thereby providing insight into soil suppressiveness), we found that *Pseudomonas* species in both suppressive and conducive soils might display similar biocontrol functions. Whole-genome sequencing proved useful to clarify taxonomy and biocontrol properties of *Pseudomonas*.

SEQUENCE ACCESSION NUMBERS

Whole-genome sequences (raw and assembled) from this study are deposited at the EBI/EMBL database under the accession number PRJEB59762, *rpoD* metabarcoding data under the accession number PRJEB61447 and *rpoD* and *rrs* gene sequences of putative fluorescent *Pseudomonas* under the accession number PRJEB64203.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ETHICAL STATEMENT

The experiments did not involve human participants and/or animals.

SUPPLEMENTARY DATA

Table S1. Most probable numbers (MPN) per root system of putative *Pseudomonas* from the rhizospheres of wheat grown in MI soils, showing 95% confidence intervals (Cornish & Fisher).

Soil	Fg1 inoculation	MPN/root system	Confidence interval (95%)	
			Lower	Higher
MI2	Inoculated	8.0×10^{7}	1.9×10^{7}	2.0×10^{8}
MI3	Inoculated	4.5×10^{7}	1.3×10^{7}	1.1×10^{8}
MI4	Inoculated	4.5×10^{8}	1.3×10^{8}	1.1×10^{9}
MI5	Inoculated	2.7×10^{7}	8.9×10^{6}	7.0×10^{7}
MI2	Control	1.7×10^{8}	5.8×10^{7}	5.0×10^{8}
MI3	Control	8.0×10^{9}	1.9×10^{9}	2.0×10^{10}
MI4	Control	4.5×10^{8}	1.3×10^{8}	1.1×10^{9}
MI5	Control	8.0×10^{9}	1.9×10^{9}	2.0×10^{10}

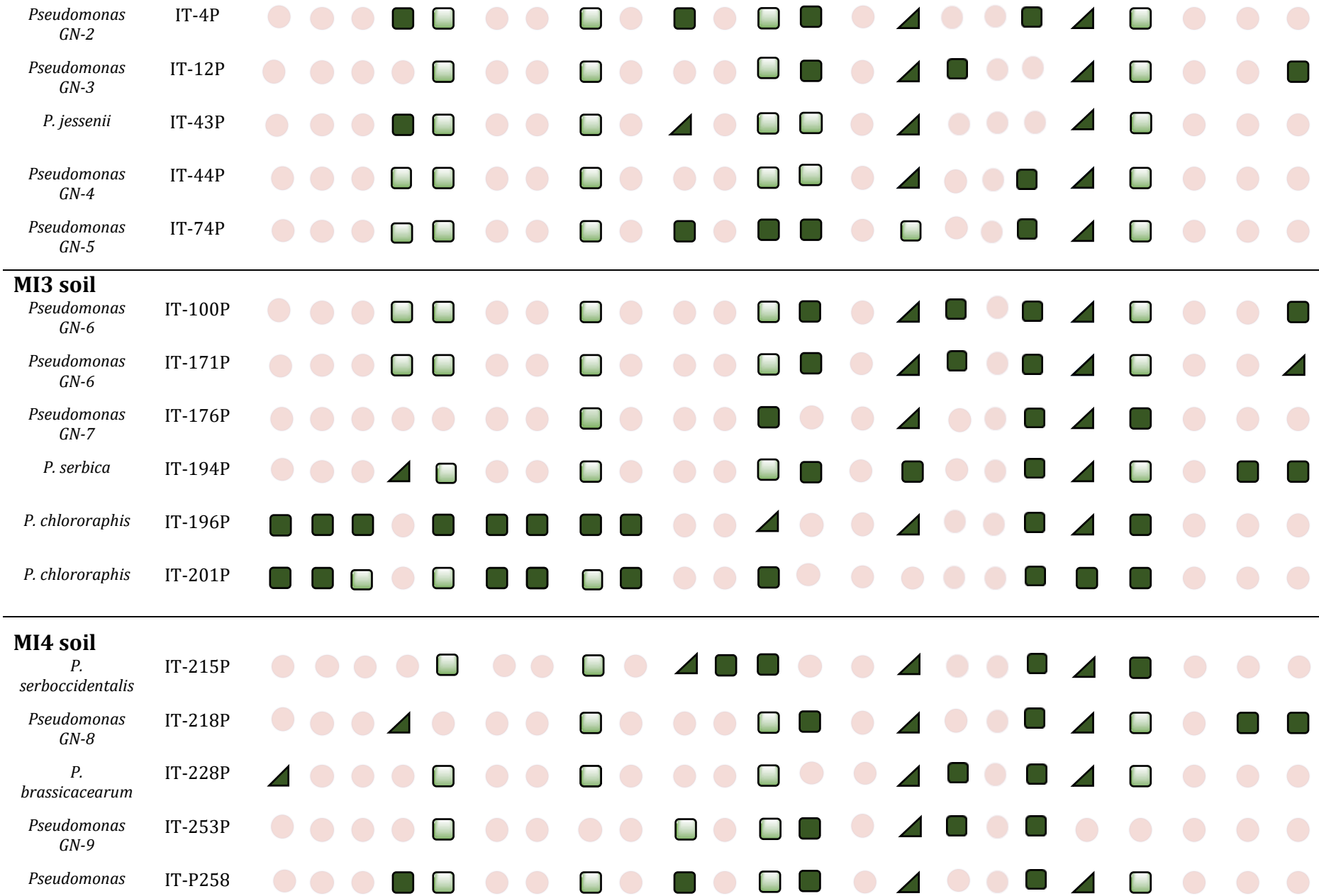
Table S2. Number of putative biosynthetic gene clusters (BGCs) and number of BGCs with completion 1 or 1*, in the *Pseudomonas* strains, found using the antiSMASH (Blin et al., 2019) within the MicroScope platform.

Soil	Species name from TYGS	Bacterial isolate	Number of putative BGCs	Number of BGCs with completion 1 or 1*
MI2	*P. siliginis*	IT-1P	8	0
	Pseudomonas GN-1	IT-2P	11	1
	Pseudomonas GN-2	IT-4P	8	0
	Pseudomonas GN-3	IT-12P	10	1
	P. jessenii	IT-43P	8	0
	Pseudomonas GN-4	IT-44P	8	0
	Pseudomonas GN-5	IT-74P	10	0
MI3	*Pseudomonas GN-6*	IT-100P	8	0
	Pseudomonas GN-6	IT-171P	9	0
	Pseudomonas GN-7	IT-176P	6	0
	P. serbica	IT-194P	12	0
	P. chlororaphis	IT-196P	15	3
	P. chlororaphis	IT-201P	14	3
MI4	*P. serboccidentalis*	IT-215P	9	0
	Pseudomonas GN-8	IT-218P	11	0
	P. brassicacearum	IT-228P	13	1
	Pseudomonas GN-9	IT-253P	7	1
	Pseudomonas GN-2	IT-P258	8	0
	Pseudomonas GN-10	IT-260P	11	1
	P. zeae	IT-265P	11	1
	Pseudomonas GN-11	IT-291P	9	0
	Pseudomonas GN-12	IT-294P	10	1
MI5	*P. chlororaphis*	IT-324P	16	2
	Pseudomonas GN-13	IT-347P	12	1
	P. marginalis	IT-357P	14	2
	P. serbica	IT-P366	11	0
	P. chlororaphis	IT-373P	15	3
	P. serboccidentalis	IT-P374	11	0
	Pseudomonas GN-14	IT-395P	11	1

* When two or more genes in a single MIBiG (The Minimum Information about a Biosynthetic Gene cluster database) curated region were similar, the same gene in MicroScope database can hit on these MIBiG genes. When this happens, the completion can be higher than 1 (represented by 1*).

Table S3. Putative biosynthetic gene clusters (BGCs) identified using antiSMASH (Blin et al., 2019) and manually curated. Dark green square shows the presence of BGC, pale green square shows the presence of BGC, but with different gene synteny, dark green triangle shows the partial presence of BGC and pale pink circle shows the absence of BGC. [a] BGC compared to the one present in IT-196P, [b] compared to IT-324P, [c] compared to IT-373P, [d] compared to IT-P258, [e] compared to IT-P4, [f] compared to IT-215P, [g] compared to IT-P374, [h] compared to IT-P366, [i] compared to IT-194P, [j] compared to *Pseudomonas ogarae* F113 and [k] as described in Costa et al. (2021). Annotation was completed on April 18th, 2023.

Bacterial isolate		Antimicrobial compounds							Siderophores					Metabolism/ cellular processes			Mot ility	Secretion systems			Mixed regions				Virulence/ pathogenesis	
		Massetolide A (cyclic lipopeptide) [a]	Pyrrolnitrin [a]	Phenazine [a]	Viscosin-like peptide synthesis [d]	Fragine-like peptide synthesis [a]	Putative bacitracin/enterobactin synthesis [a]	Achromobactin-like siderophore synthesis [a]	Pyoverdine-like siderophore synthesis 1 [a*]	Pyoverdine-like siderophore synthesis 2 [a*]	Rhizobactin-like siderophore biosynthesis [d]	IucA/IucC family siderophore biosynthesis [f]	Aryl polyene [a]	Cellular processes, signalling, signal transduction [e]	Cell wall synthesis/lipid metabolism [b]	Biofilm formation/chemotaxis [i]	Type III secretion system [j]	Type IV secretion system (Dot/Icm family) [k]	Type VI secretion system [j]	Pyoverdine synthesis/catabolism of citronellol and geraniol [c]	Lipoteichoic acid synthesis; putative cyanophycin synthesis-like [a]	Genes for lipid biosynthesis, signaling molecules and penicilin amidase [g]	Steroid degradation [h]	Potentially involved in bacterial pathogenesis: membrane sulfatase and ubiquitin transferase [h]
Reference strains																										
P. ogarae	F113	●	●	●	●	■	●	●	■	●	●	●	■	●	●	◢	■	●	■	◢	■	●	●	●		
P. protegens	CHA0	●	●	●	●	●	●	●	■	●	●	●	■	●	●	◢	●	●	■	◢	■	●	●	●		
MI2 soil																										
P. siliginis	IT-1P	●	●	●	●	■	●	●	■	●	●	●	■	■	●	◢	●	●	■	◢	■	●	●	●	■	
Pseudomonas GN-1	IT-2P	◢	●	●	●	■	●	●	■	●	◢	■	■	■	●	◢	●	●	■	◢	■	●	●	●	■	


Pseudomonas GN-2 IT-4P
Pseudomonas GN-3 IT-12P
P. jessenii IT-43P
Pseudomonas GN-4 IT-44P
Pseudomonas GN-5 IT-74P
MI3 soil
Pseudomonas GN-6 IT-100P
Pseudomonas GN-6 IT-171P
Pseudomonas GN-7 IT-176P
P. serbica IT-194P
P. chlororaphis IT-196P
P. chlororaphis IT-201P
MI4 soil
P. serboccidentalis IT-215P
Pseudomonas GN-8 IT-218P
P. brassicacearum IT-228P
Pseudomonas GN-9 IT-253P
Pseudomonas IT-P258

GN-2		
Pseudomonas GN-10	IT-260P	
P. zeae	IT-265P	
Pseudomonas GN-11	IT-291P	
Pseudomonas GN-12	IT-294P	
MI5 soil		
P. chlororaphis	IT-324P	
Pseudomonas GN-13	IT-347P	
P. marginalis	IT-357P	
P. serbica	IT-P366	
P. serbica plasmid	IT-P366	
P. chlororaphis	IT-373P	
P. serboccidentalis	IT-P374	
Pseudomonas GN-14	IT-395P	

* Pyoverdine-like siderophore synthesis region 1 is present in *P. chlororaphis* IT-196P from position 4223142 to 4276158 and pyoverdine-like synthesis region 2 from position 6268542 to 6339315.

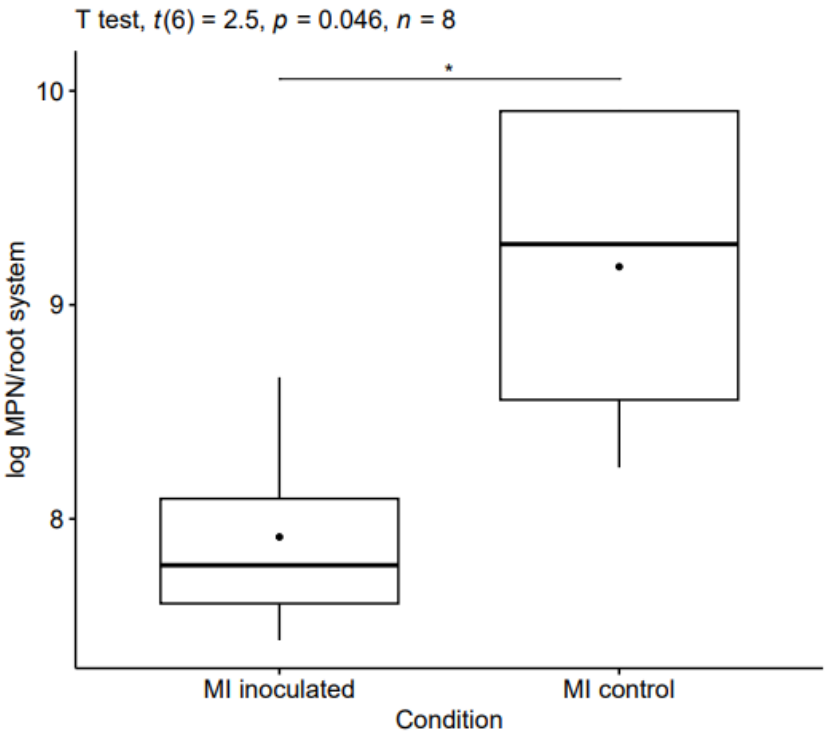


Figure S1. Log_{10} MPN of putative fluorescent *Pseudomonas* per root system of wheat plants grown in MI soils inoculated or not with *Fusarium graminearum* Fg1. Data were compared with Student's *t* test, at $P < 0.05$.

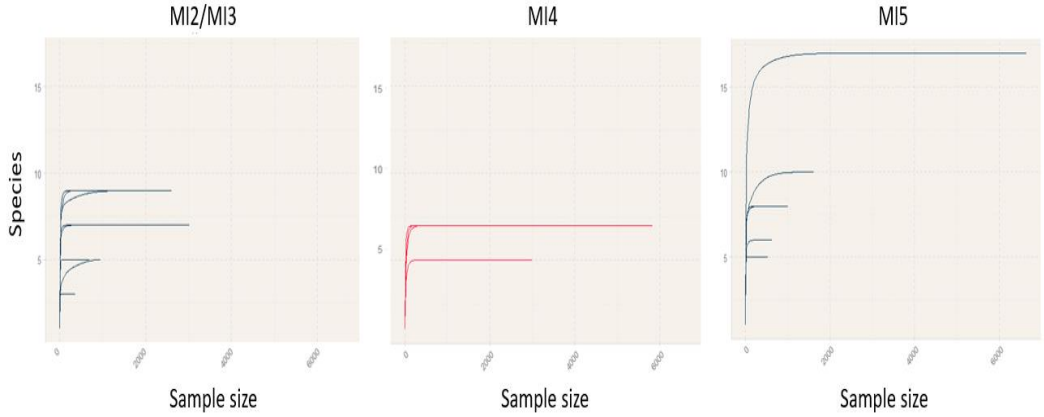


Figure S2. Rarefaction curves with the estimated species richness of each replicate for *rpoD* metabarcoding of MI2/MI3, MI4 and MI5 soil categories. One MI2 and one MI3 replicate (with too many reads), and one MI2, one MI4 and one MI5 replicate (with not enough reads) were discarded. Rarefaction curves for MI2/MI3 category are presented as joint samples MI2 and MI3.

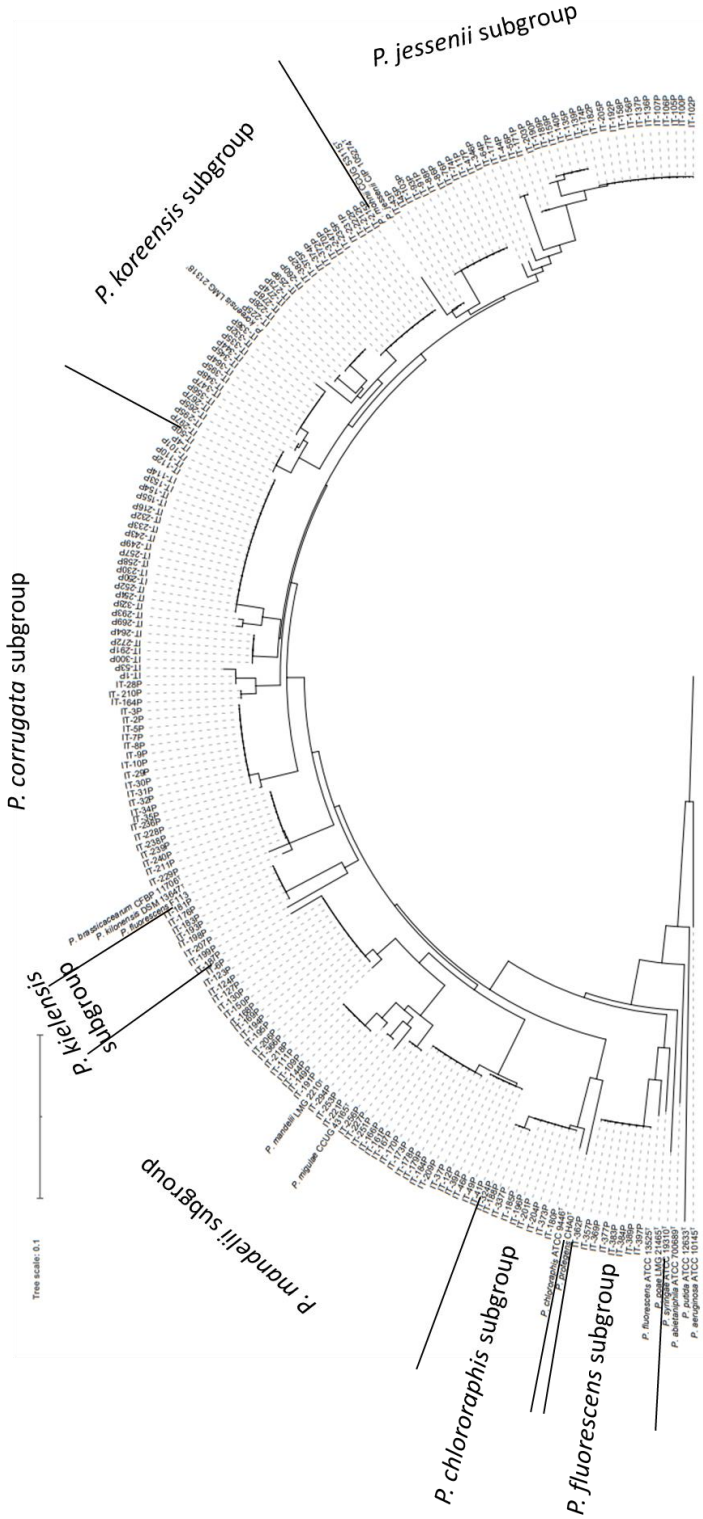


Figure S3. Phylogenetic tree of 185 *Pseudomonas* isolates characterized based on their *rpoD* gene sequence, and belonging to the *Pseudomonas fluorescens* group. The phylogenetic tree includes 14 *Pseudomonas* type strains (Garrido-Sanz et al., 2016) and *Pseudomonas aeruginosa* ATCC 10145^{T}, used for tree rooting. Other *Pseudomonas* groups such as the *Pseudomonas syringae*, *Pseudomonas putida* and *Pseudomonas lutea* groups are also represented. Analysis was done using the SeaView multiplatform (Gouy et al., 2010), the sequences were aligned with Muscle5 v.3.8.31 (Edgar, 2022), the phylogenetic tree was constructed with Distance method and 1000 bootstraps and visualized using iTol (Letunic & Bork, 2021).

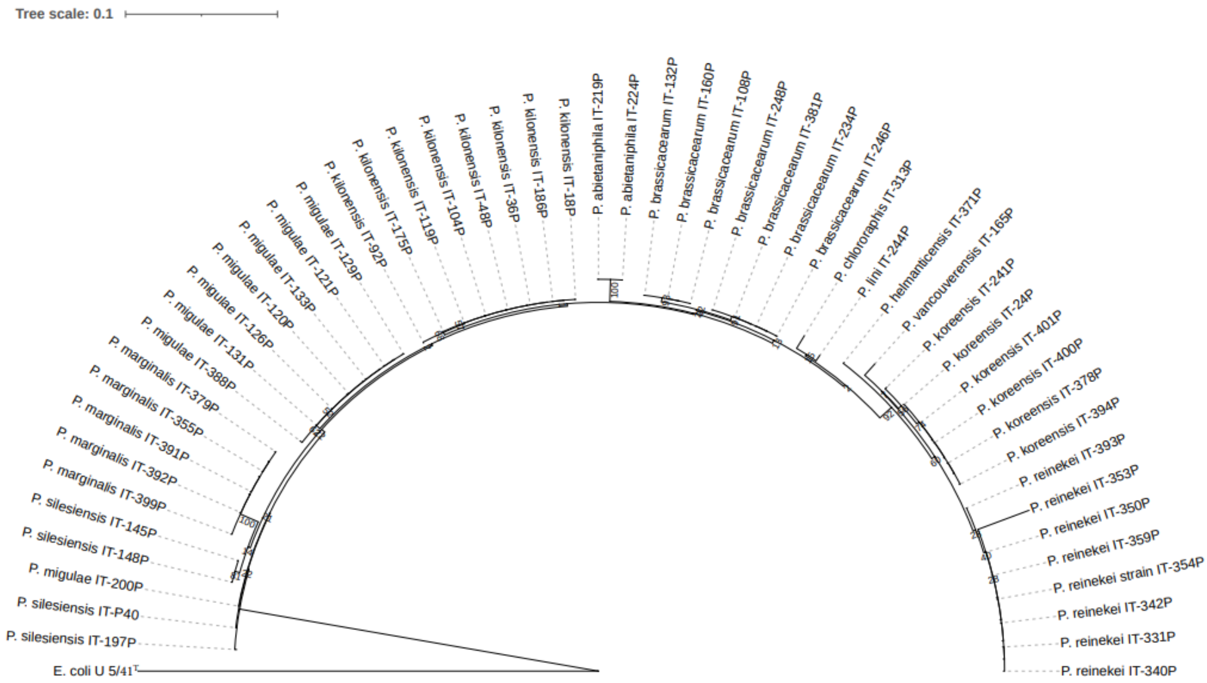


Figure S4. Phylogenetic tree of putative *Pseudomonas* isolates characterized based on their 16S rRNA gene *rrs*. *E. coli* U 5/41^T was used for tree rooting. Analysis was done using the SeaView multiplatform (Gouy et al., 2010), the sequences were aligned with Muscle5 v.3.8.31 (Edgar, 2022), and phylogenetic tree was constructed with Distance method and 1000 bootstraps and visualized using iTol (Letunic & Bork, 2021). Isolates were identified using the GenBank database and BLAST option (based on hits with very high query coverage and percent identity).

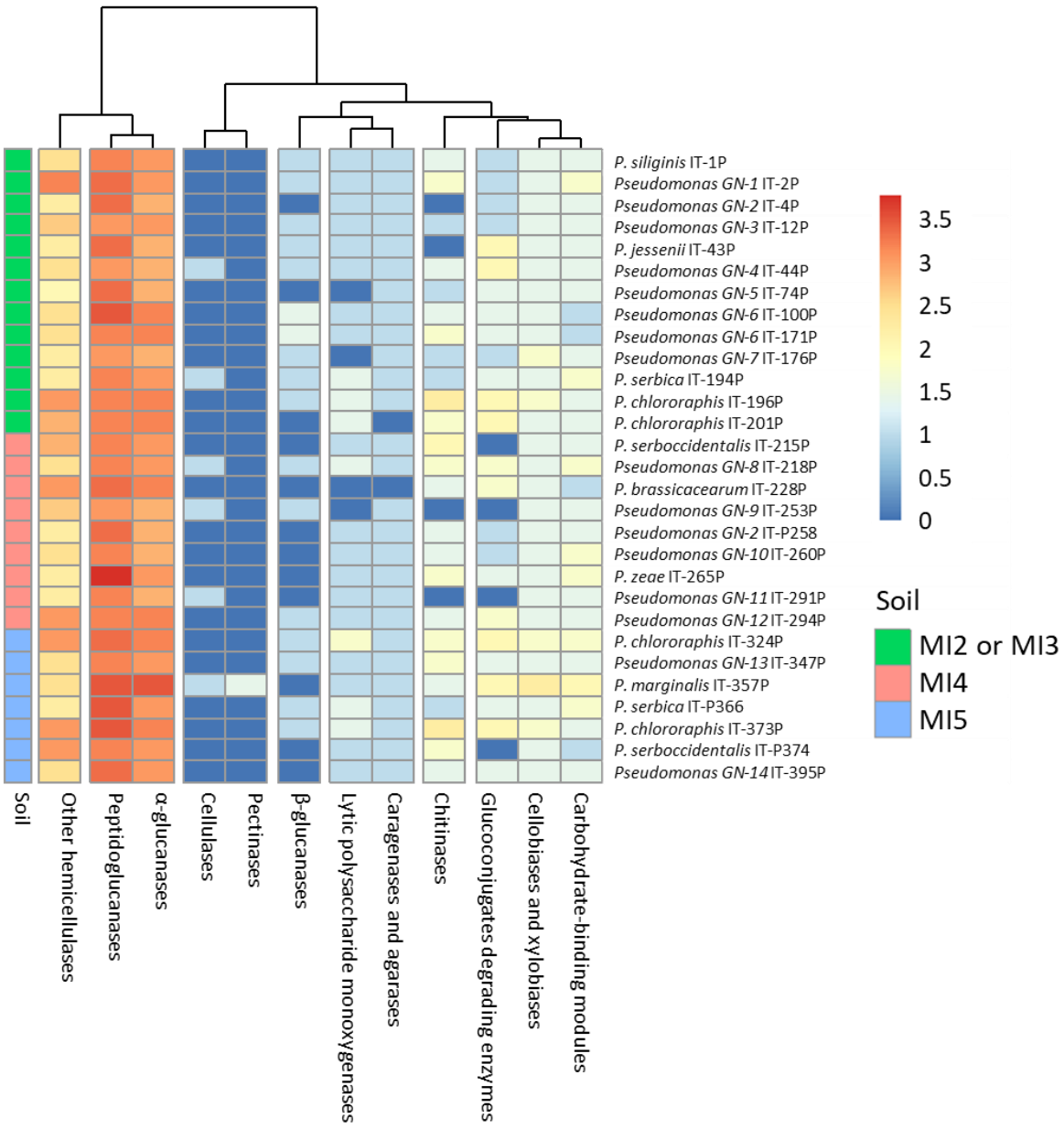


Figure S5. Heatmap showing the abundance of CAZyme genes annotated for each function found in the genomes of *Pseudomonas* strains. Legend shows transformed counts.

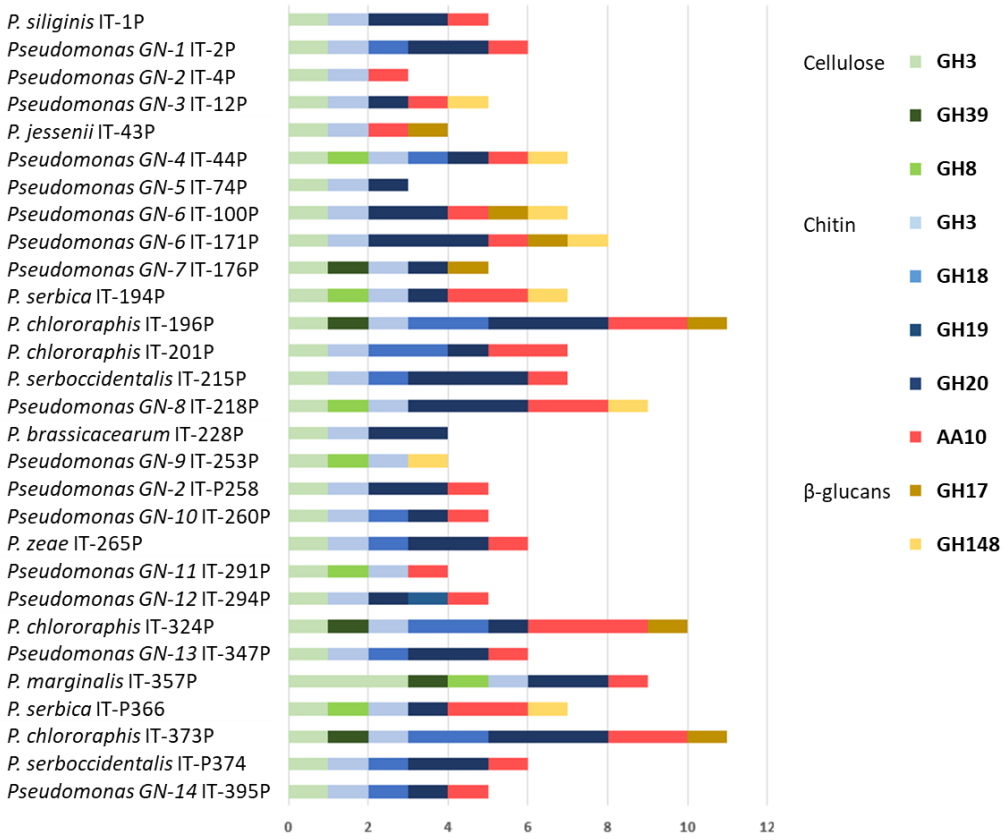


Figure S6. Abundance of *Pseudomonas* genes corresponding to CAZyme families potentially targeting cell wall components in fungi and oomycetes (cellulose, chitin and β-glucans).

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GENERAL DISCUSSION

Cereal grains (wheat, corn, rice, barley, sorghum, oats and rye) are the most produced crops worldwide, substantially supplying energy to humans and livestock (Fatima et al., 2020). For example, in 2018, wheat provided 20% of all the calories consumed by humans ("Food and Agriculture Organization of the United Nations," 2020). In the last decade, with growing human population and demand for cereal grains, agricultural management has become increasingly intensified, with excessive use of fertilizers and pesticides, leading to declined soil microbial diversity. These factors, together with the ongoing climate change, have paved a way for even higher intrusion of pests and diseases, which can spread quickly and cause significant yield losses (Ramankutty et al., 2018). Wheat, for example, experiences up to 23% yield loss due to pests and pathogens (Savary et al., 2019), with *Fusarium graminearum* as one of the predominant pathogens causing increased damage to wheat (Yli-Mattila, 2010; Nielsen et al., 2012; West et al., 2012). As in many European countries, in Serbia, agroecological conditions are favorable for development of phytopathogenic and mycotoxicogenic *F. graminearum* (Stanković et al., 2008; Obradović et al., 2017). In the context of global climate warming, the relationship between biodiversity and crop health has received more attention with the emergence of different pathogens/pests (Trębicki et al., 2017). By exploring the existing mechanisms underlying soil suppressiveness, and trying to learn from Nature, we are given an opportunity to invent nature-based strategies to control fungal pathogens. In this thesis, we explored the relationship of manure amendments and fungistasis/suppressiveness to *F. graminearum*, as well as compared the total prokaryotic and fungal communities residing in suppressive vs. non-suppressive soils (Chapter 2). Moreover, we tested the usefulness of fungistatic soils as a source of biocontrol agents against *F. graminearum* (Chapter 3), and lastly, we identified the genomic and functional particularities of *Pseudomonas* bacteria isolated from suppressive vs. non-suppressive soils, and described two novel *Pseudomonas* species (Chapter 4). The main findings of Chapters 2, 3 and 4 are summarized in Figure 1.

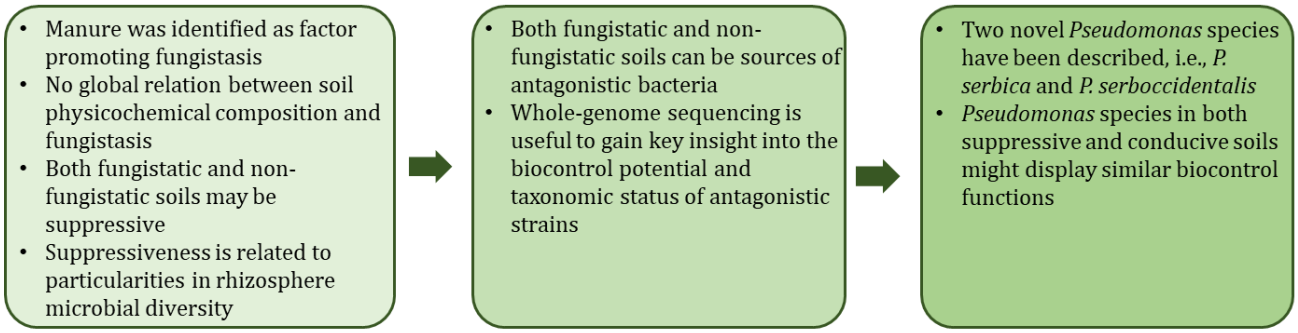

Figure 1. Main findings from the experimental Chapters 2, 3 and 4 presented in this PhD thesis.

Fungistasis and soil suppressiveness to *Fusarium graminearum* damping-off disease of wheat

Soils naturally suppressive to soil-borne pathogens have been recognized worldwide more than 70 years ago (Vasudeva & Roy, 1950; Alabouvette, 1986; Schlatter et al., 2017; Lv et al., 2023). However, in many countries of the world and for the majority of soil-borne pathogens, the distribution of disease suppressiveness is undetermined because of the absence of simple tools that will enable reliable identification of such soils. Fungistasis, a term used to describe competition and antagonism of the entire soil microbiota and pathogenic fungal propagules, significantly contributes to disease suppressiveness by decreasing the amount of fungal inoculum available for disease development in plants (Garbeva et al., 2011). In the Chapter 2 of this thesis, we sampled 26 manured and non-manured soils from different locations in

Serbia, i.e., from locations in northern, plain part of Serbia, where the agriculture is more intensive because of the existence of fertile soil of type chernozem, and in western/central, hilly parts of Serbia, where the agriculture is more traditional (Tanasijević et al., 1964; Nejgebauer et al., 1971). Firstly, these 26 soils were screened for fungistasis to *F. graminearum*, as this mycotoxicogenic and economically important pathogen is responsible for significant wheat yield losses (West et al., 2012). We found fungistasis potential in 10 of the screened soils (38%), where the amount of fungal DNA decreased and all of these soils were from western/central Serbia, where the agricultural practices are more oriented towards biodiversity protection. Contrarily, none of the fungistatic soils were found in northern parts of Serbia (soils SO and NK), where soils are of type chernozem and agriculture is more intensive. This could be due to long-term agricultural exploitation of these soils (despite using manure amendments at some fields), that led to disturbed soil microbial diversity and higher infestation after inoculating *F. graminearum.* Out of these 10 fungistatic soils, 7 of them (70%) had been amended with manure, and manure treatments have been targeted as significant factor determining fungistasis, especially in soils from Mionica (MI). This is in line with research by Legrand et al. (2019), that showed positive relation between manure amendments and fungistasis potential. However, no significant relation was found between fungistasis and soil physicochemical properties, except that MI (fungistatic) soils contained higher organic matter and potassium contents, compared to non-fungistatic MI soils. MI soils were further used to perform suppressiveness assay towards *F. graminearum* damping-off disease of wheat, and to our knowledge, this was the first study that had two-fold approach and performed both fungistasis and *in planta* suppressiveness assay (Chapter 2). Fungistasis and suppressiveness assay results matched for 3 out of 4 MI soils, i.e., soils MI2 and MI3 were both fungistatic and suppressive, soil MI4 was non-fungistatic and non-suppressive, whereas soil MI5 was non-fungistatic and suppressive. Moreover, it was observed that wheat shoot biomass, length and density were overall higher in manure-amended soils, as found before (Ibrahim et al., 2008), and this is also in line with previous research that highlight the significance of organic and compost amendments in enhancing soil phytoprotection capacity against soil-borne pathogens (Mousa & Raizada, 2016; Mitsuboshi et al., 2018; Nguyen et al., 2018).

Further, we chose rhizospheres of wheat plants grown in soils MI2 (fungistatic and suppressive), MI4 (non-fungistatic and non-suppressive) and MI5 (non-fungistatic and suppressive) to look for fungal and prokaryotic populations that make a distinction between the three soil categories, so we performed ITS and 16S rRNA-based taxonomic profiling. However, our results showed that there is no specific fungal or prokaryotic group that is enriched in suppressive soils, and that microbiota was soil-specific. Although at all fields, wheat was grown in a crop rotation, at the time of the sampling MI soils for fungistasis assay, fields were grown with alfalfa (MI2), meadow (MI5) or wheat (MI4), while at the time of the sampling MI soils for suppressiveness *in planta* assay, fields were grown with maize (MI2), wheat (MI5) or left as meadow (MI4). In the case of MI5 soils, as wheat was grown at the time of soil sampling (spring 2021) for suppressiveness assay and ITS and 16S rRNA metabarcoding analysis, it is possible that this contributed to the enrichment of microbial populations suppressing *Fusarium* damping-off disease of wheat, compared to fungistasis assay, where at the time of soil sampling (autumn 2020) there was a meadow. These differences between the two soil samplings (different timing and different crops present in the fields) likely contributed to the difference between the microbiota. It is well known that plant microbiota is acquired from the surrounding soil environment and that plants recruit their microbiota with their exudates, that are determined by plant species and variety, and developmental stage (Sánchez-Cañizares et al., 2017). Studies also suggested that microbiota can be shaped by the soil properties (i.e., availability of nutrients and carbon, pH; Custódio et al., 2022) and evolutionary history of plants (Bouffaud et al., 2016; Simonin et al., 2020).

Furthermore, microbes and microbial communities are constantly evolving and adapting to dynamically changing ecological and biotic conditions, in order to survive, the latter being defined as the "Red Queen hypothesis" (Van Valen, 1977). As shown in our study (Chapter 2), when biotic conditions in rhizosphere soils were changed due to seed inoculation with *F. graminearum,* the rhizosphere prokaryotic and fungal community also changed, probably due to antagonistic interactions between the resident microbiota and the added pathogen and/or changes in plant metabolism and exudates (Rojas et al., 2014). This change in quantity and composition of plant root exudates due to pathogen inoculation is termed "cry for help" strategy, when plants recruit microbes with biocontrol properties (Rizaludin et al., 2021). For example, it was shown that inoculation of barley plants with *F. graminearum* triggered changes in root exudates, where roots started producing different antifungal organic acids (Lanoue et al., 2010), that also act as attractants for fluorescent *Pseudomonas*, and in such way, barley plants manipulated their rhizosphere microbial community composition (Oku et al., 2014). Similarly, inoculation of *Carex arenaria* plant with *F. culmorum*, provoked changes in composition of Volatile Organic Compounds (VOCs) produced by plant roots, and attracted microbes with antifungal properties (Schulz-Bohm et al., 2018). This dynamic system that consists of the holobiont (plant with its microbiome), phytopathogen and surrounding environmental factors determine the suppressive nature of soils, as defined by Jayaraman et al. (2021) with his disease triangle concept. Another issue is that in the natural ecosystems, it is rarely one pathogen that has to be controlled, rather there is the entire myriad of soil-borne pathogens. For example, there was an attempt to modify the soil microbiota with the *Brassica napus* seed meal amendments, aiming to induce suppressiveness towards apple replant disease caused by the phytopathogen *Rhizoctonia solani*. This approach successfully suppressed *R. solani*, but increased populations of *Pythium* spp. (Mazzola, 2007). Ideally, in order to profoundly understand soil suppressiveness to soil-borne diseases, future research should study all three factors from the triangle concept in parallel, aiming to better understand correlation between these factors and disease suppression. These data might provide a clue how the soil microbiome, that serves as a source of microbes for the plant microbiome, can be manipulated, aiming to achieve soil suppressiveness that is customized to the plant, pathogen(s) and the surrounding environmental factors.

What if we go outside the lab?

In Chapter 3, we isolated 244 bacteria of contrasting taxonomy, from the rhizosphere of plants grown in fungistatic and non-fungistatic soils of Mionica and Čačak, aiming to asses the usefulness of fungistatic soils as sources of bacteria with antagonistic properties towards *F. graminearum*. These bacteria were tested in a dual confrontation assay with *F. graminearum*, yielding similar number of bacteria from both fungistatic and non-fungistatic soils, 23 in total, as to make a conclusion that not only fungistatic soils are a good source of potential antagonistic bacteria. Analysis of the 23 antagonistic bacteria showed that genomic and functional profiles of strains from both fungistatic and non-fungistatic soils were similar, and taxa-specific. However, when a plant phytoprotection assay was performed, only one isolate - *Pseudomonas GS-5* IT-194MI4 (from non-fungistatic soil) enhanced wheat germination and conferred protection from crown-rot disease, but at the expense of shoot biomass and chlorophyll rate. On the contrary, isolate *Brevibacillus GS-3* IT-7CA2 inhibited *F. graminearum* Fg1 mycelial growth for 95% in *in vitro* dual confrontation assay, but in *in planta* assay, this strain contributed to even lower seed germination, compared to *F. graminearum* Fg1-inoculated control. This was not surprising, as it was already shown that many microbes that perform well in *in vitro* conditions, fail in greenhouse *in planta* and field experiment (Comby et al., 2017; Besset-Manzoni et al., 2019). But why does this happen?

Most of the phytoprotection studies select microbes based on *in vitro* assays, where pathogens are directly inhibited by the antagonistic microbe, but in the system plant-phytopathogen-antagonistic microbe, there are other mechanisms that may take place. For example, it is known that microbes can enhance plant defenses by inducing systemic resistance upon the pathogen attack (Magotra et al., 2016), or can enhance plant fitness, i.e., by increasing the bioavailability of potassium, phosphorus, nitrogen, iron and other essential minerals (Kızılkaya, 2008; Rasouli-Sadaghiani et al., 2014; Dasila et al., 2023), and/or by increasing plant resistance to stresses caused by heavy metals, drought and increased salinity (Glick, 2012). In such a way, interactions that take place when the plant is present in the system are more complex than when interactions are observed solely on the pathogen-microbe level and choosing microorganism for the plant assay based on one *in vitro* experiment may be challenging and misleading (Besset-Manzoni et al., 2019). Comby et al. (2017) performed a two-fold screening study of wheat endophytes that could potentially protect wheat from Fusarium Head Blight (FHB), i.e., they performed a classical dual-culture assay, and they checked the ability of endophytes to protect wheat spikelets from disease, and the results between the two approaches were different, raising the question of the most suitable screening approach. Nonetheless, factors such as host plant compatibility, or inoculation method (seed-coating, root-diping, foliar or soil inoculation) largely impact the successs of bacterial inoculants. For example, it is known that some bacteria act as bioherbicides, thus contributing to reduction of seed germination and plant growth (Fang et al., 2022), while different methods of inoculation may also largely affect the outcome (Stoll et al., 2021). One limiting factor of our study (Chapter 3) is that soil used for the *in planta* experiment was sterilized, and free from other microorganisms that might antagonize our antagonistic bacteria tested, and conditions, i.e., temperature and lighting were controlled, which is unlike the conditions present in the field. In ideal case, after the greenhouse *in planta* assay, a field assay should be performed, as it was shown that only few microbes that performed well in *in vitro* and greenhouse *in planta* experiments, were also successful in field conditions, with changing environmental conditions (Pliego et al., 2011). One of the possible solutions to avoid this discrepancy between the different assays, would be to use microbial consortia made from multiple microorganisms with different modes of action (Nadeem et al., 2013; Besset-Manzoni et al., 2018). Although there are pieces of evidence that the consortia activity is higher in the greenhouse *in planta* assay, than in field assays, it still performs better than single-bacteria inoculants (Liu et al., 2023). However, as plants may modulate microbial metabolism and the microbial community, there is an urge to better understand plant-microbe interactions and to use that knowledge to formulate consortia with high survival in natural conditions and high plant compatibility (Maciag et al., 2023).

Describing new species found in Serbia

In Chapters 3 and 4, after the whole genome sequencing of isolates, and performing Digital DNA-DNA hybridization (dDDH) (and obtaining values below 70%, a threshold recognized for species delineation (Chun et al., 2018)), we identified so-far undescribed bacterial species, i.e., one *Bacillus*, one *Chryseobacterium*, one *Brevibacillus*, one *Burkholderia* and four *Pseudomonas* in Chapter 3, as well as 16 *Pseudomonas* in Chapter 4. In the first part of Chapter 4 (published; Oren & Goker, 2023; Todorović et al., 2023), we described characterization of the two newly identified *Pseudomonas* species, and their morphological, genomic, biochemical and physiological features, and we proposed names *P. serbica* and *P. serboccidentalis*, following the guidelines of International Code of Nomenclature of Prokaryotes (Oren et al., 2023).

However, a substantial number of species remain undescribed during the course of this thesis, therefore this would require future steps aiming to describe them. Considering that the whole genome sequencing of these species has already been performed, Average Nucleotide

Identity (ANI) analysis would have to be done, and to confirm that the obtained ANI values between novel species and the closest described species present in the public databases are below 95% (Chun et al., 2018). Furthermore, species would have to be deposited in at least two culture collections in two different countries, as required by International Journal of Systematic and Evolutionary Microbiology (microbiologyresearch.org). Finally, these novel species would have to be characterized phenotypically, if they are to be fully described.

Pseudomonas in suppressive vs. non-suppressive soils

In the second part of Chapter 4, we isolated 406 putative *Pseudomonas*, from the rhizospheres of wheat plants grown in suppressive and non-suppressive soils, that had been previously inoculated or not with *F. graminearum*. Amplification of the *rpoD* gene was successful with 185 of them, yielding 65 *Pseudomonas* with different *rpoD* sequences, and 29 of them were chosen for the whole-genome sequencing. These 29 sequenced *Pseudomonas belonged* to the *P. fluorescens* group, i.e., *P. fluorescens*, *P. kielensis*, *P. mandelii*, *P. jessenii*, *P. koreensis*, *P. corrugata* and *P. chlororaphis* subgroups. *Pseudomonas* from the fluorescent group have been extensively studied in the case of Fusarium wilt, notably in southern France (Alabouvette, 1986) and in California (Scher & Baker, 1980), where main mechanisms underlying this disease suppression were found to be synthesis of phenazine (Mazurier et al., 2009) and competition for iron (Scher & Baker, 1980) (Chapter 1). In this study, comparison of genomic and functional properties of *Pseudomonas* isolated from suppressive and non-suppressive soils revealed no significant differences, i.e., all specificities were taxonomy related, although metabarcoding analysis of the *Pseudomonas* community revealed soil-specific composition. However, real biocontrol capacities of microbes are sometimes difficult to predict in lab conditions. For example, although plant protection that takes place in suppressive soils may be a result of action of one or a few microbial populations, it may be that other microbial community members have an important role on the former, i.e., that they influence their root colonization or biocontrol gene expression (Kyselková & Moënne-Loccoz, 2012). Moreover, 2,4-diacetylphloroglucinol (DAPG)-producing *P. protegens* strains are found in both soils suppressive and conducive to black root-rot disease of tobacco, but their phytoprotective capacities differ. It was shown that this was due to the presence of iron-releasing minerals in suppressive soils, that alter iron bioavailability and positively impact the expression of DAPG genes in suppressive soils. This was confirmed by adding iron to conducive soils, which resulted in enhanced expression of DAPG genes in these soils (Almario et al., 2013). Therefore, the next stage in defining particularities between disease suppressive and non-suppressive soils would be to assess the levels of expression of biocontrol genes. Another point is that population of biocontrol strain has to achieve a certain threshold in order to achieve phytoprotection (Weller et al., 2007). For example, it is known that non-pathogenic *F. oxysporum* Fo47 is needed in concentrations 10 to 10^2 times higher than the phytopathogen itself, in order to suppress the pathogenic *F. oxysporum* (Fravel et al., 2003). Similarly, it was observed that *Pseudomonas defensor* (ex *fluorescens*) WCS374 has the ability to suppress Fusarium wilt, but only if present at $\sim 10^5$ CFU per g of root (Raaijmakers et al., 1995). Therefore, it would be useful to assess the relative abundance of different *Pseudomonas* genotypes. Overall, analysis of microbial communities in soils of contrasted suppressiveness status seems promising approach in an attempt to identify taxa that are more abundant or whose genes are more expressed in suppressive soils, as those taxa might represent potential plant-protecting microbes (Benítez & McSpadden Gardener, 2009; Pliego et al., 2011).

Secondary metabolites in soil suppressiveness to *Fusarium graminearum*

Microorganisms are able to produce a wide variety of secondary metabolites, including antibiotics and VOCs, that are not involved in primary metabolism, but rather they help microbes to harvest nutrients and to interact and communicate with other microorganisms, including competitors and symbionts (Macheleidt et al., 2016). Secondary metabolites have small molecular weight and they are very structurally heterogeneous, with enormous potential still being unraveled (Keswani et al., 2020). Several identified secondary metabolites have already been linked to disease suppressiveness, such as production of thiopeptide by *Streptomyces* (Cha et al., 2016), phenazines by *Pseudomonas* (Mazurier et al., 2009), and production of iturin C, bacillomycin, fengycin by *Bacillus licheniformis* (Yadav et al., 2021) in the case of Fusarium wilt, as well as production of DAPG by *Pseudomonas* in the case of take-all disease of barley and wheat (Weller et al., 2007; Chapter 1). In Chapters 3 and 4 of this thesis, several putative biosynthetic gene clusters (BGCs) have been identified in both biocontrol and *Pseudomonas* strains, that could be potentially involved in suppression of *F. graminearum* and damping-off disease. On top of that, several biocontrol and *Pseudomonas* strains (Chapters 3 and 4) *in vitro* produced VOCs with antagonistic properties against *F. graminearum,* but this would require further identification of exact VOCs that act antagonistically.

The BGCs found in strains from this study (Chapter 3 and 4) mostly encoded for siderophores, lipopeptides and polyketides, groups of metabolites that are widely known for their antifungal properties (Chen et al., 2009; Esmaeel et al., 2016). However, presence of certain BGC in the bacterial genome, does not necessarily mean that the corresponding metabolite is indeed synthesized and excreted in the rhizosphere, moreover as the BGC expression is often determined by the surrounding abiotic and biotic conditions (Dastogeer et al., 2020). Therefore, tools such as transcriptomic, proteomic and metabolomics studies, as well as the use of reporter genes, such as the *Green Fluorescent Protein* (GFP) can help in elucidating the production of secondary metabolites encoded in the bacterial genome (Kiely et al., 2006; Barret et al., 2009; Mavrodi et al., 2021). For example, with the genome mining it was shown that *Bacillus cabrialesii* TE3T contains BGCs coding for production of several secondary metabolites, and with metabolomic techniques it was shown that only surfactin, fengycin, and rhizocticin A are indeed produced and have antifungal activity against phytopathogen *Bipolaris sorokiniana*, a causal agent of spot blotch disease of durum wheat (*Triticum turgidum* L. subsp. *durum*) (Villa-Rodriguez et al., 2021). Another issue is that versatile genetic diversity of BGCs in living organisms, together with limited verified databases that could help in encoding the exact function, leaves only the possibility to assume their exact function. In order to verify if the certain BGCs found in bacterial genome have a role in disease suppression, it would require to perform a site-directed mutagenesis, and with mutated strain and wild type, it would be possible to compare them for their antifungal properties (Wang et al., 2020). Similar research was already conducted by Mendes et al. (2011), when they performed transposon mutagenesis on a gene cluster encoding for thanamycin synthesis in strain *Pseudomonas* sp. SH-C52, obtained from suppressive soils, and showed that the mutant has the ability to colonize the rhizosphere of sugar beet seedlings, but could not protect it from *R. solani* infection like the wild type. In a similar manner, our findings about biocontrol and *Pseudomonas* strains and putative BGCs found in their genomes might be validated and their function can possibly be linked to fungistatic or suppressive soil status.

Concluding remarks and future perspectives

Overall, the research conducted during this thesis presents the first screening of fungistasis to *F. graminearum* across 26 contrasting fields in Serbia and screening of suppressiveness to

F. graminearum damping-off disease of wheat at the same time. Here we also tested the impact of soil physicochemical properties and manure amendments on fungistasis, and concluded that there is no global relationship between soil physicochemical composition and fungistasis, while manure amendments may promote fungistasis (Chapter 2). Furthermore, we tested the usefulness of fungistatic soils as a source of biocontrol agents against *F. graminearum*, and concluded that they are as useful as non-fungistatic soils (Chapter 3). Then, we performed comparison of *Pseudomonas* strains isolated from suppressive vs. non-suppressive soils and found that their genomic and functional potential is rather taxonomy than soil-specific, and we used *rpoD* primers of Manriquez (2021) for the first time to reveal *Pseudomonas* populations in suppressive vs. non-suppressive soils (Chapter 4). Finally, we described two novel *Pseudomonas* species, *P. serbica* and *P. serboccidentalis* (Chapter 4). In general, this thesis outlines the microbial nature of suppressive soils, with dynamic interactions taking place between numerous actors in the rhizosphere.

This thesis can serve as a foundation for further research on soils suppressive to *F. graminearum* diseases and a base for rhizosphere microbiome studies, and it adds up to the research already conducted on soils suppressive to *Fusarium* diseases in other parts of the world, in different climatic conditions. As in this thesis, fungistasis and suppressiveness assays were performed at Serbian locations of contrasting landscape, soil types, and agricultural practices, we suggest implementing this approach in future research, as this might help decipher the occurrence patterns of these phenomena. As a future direction, we propose the implementation of metatranscriptomics and metabolomics in research on suppressive soils, in order to check for differences in plants grown in suppressive vs. non-suppressive soils. Based on the results presented in Chapter 3, it would be useful to perform a field study with potential biocontrol agent *Pseudomonas GS-5* IT-194MI4 to check for its viability in natural environment and to try different method of inoculation for other strains. This might help in targeting actors that may be used in designing a consortia able to protect plants from *F. graminearum* diseases. Another goal would be to describe newly found species from Chapters 3 and 4. Since there were no particularities that made a distinction between *Pseudomonas* found in suppressive vs. non-suppressive soils, it would be of interest to check expression of genes involved in biocontrol or to check for relative abundance of different *Pseudomonas* genotypes. This study also pinpointed the metabolic potential of wheat microbiome to produce various metabolites, including secondary metabolites, that may be important in soil suppressiveness to *F. graminearum* diseases, but this aspect needs further validation. In general, deep understanding of mechanisms underlying soil suppressiveness may help in inducing suppressiveness at sites where crops are severely attacked by *F. graminearum*, as well as by other detrimental pathogens.

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BIOGRAPHY

Irena Todorović was born on April 27th, 1995 in Belgrade, Republic of Serbia. She graduated from Zemun High School in 2014 and that same year she was enrolled in Bachelor studies at the Faculty of Biology, University of Belgrade, study program Biology, module Ecology. She graduated on September 17th, 2018, with an average score of 9.09/10, earning the title Graduated biologist. During her undergraduate studies, she was Coordinator of the Congress of Biology Students "Simplast" and Deputy president of the Student's Parliament. She was also engaged in vocational student practices, as well as in various other informal education practices.

During the 2018/19 school year, she was enrolled in the Master's Program at the Faculty of Agriculture, University of Belgrade, study program Food Technology, module Food and Environmental Microbiology. She completed this Master's Program with an average score of 9.67/10, and she defended her master thesis titled "Possibilities for application of *Bacillus megaterium* inoculum and *Aspergillus piperis* metabolites in seed biopriming" on July 4th, 2019 with grade 10/10, earning the title Master engineer of technology.

During the year 2019/2020, she was granted a French Government Scholarship for a joint PhD program at University Claude Bernard Lyon 1 (Doctoral school Évolution Écosystèmes Microbiologie Modélisation) and University of Belgrade (Faculty of Agriculture, study program Agricultural sciences), as well as "Dositeja" Scholarship from the Foundation for Young Talents in the Republic of Serbia, for the best students studying abroad. Irena Todorović speaks, reads and writes English (C2/C2 level) and French (B2/C2 level) language.

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