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RESEARCH ARTICLE

Environmental isolates of *Pseudomonas* spp. inhibit *Armillaria mellea* and promote plant growth through microbiome-mediated effects

Meriem Miyassa Aci · Giovanni Enrico Agosteo · Gabriele Pelle · Antonino Malacrinò · Leonardo Schena

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Abstract

Background and aims The management of soil-borne plant pathogens is a significant challenge in modern agriculture, particularly given the limitations of chemical control strategies. In this context, the management of the soil microbiome can be an important tool towards the biological control of soil-borne plant pathogens. In this study, we isolated bacterial strains from soil microbiomes with potential biocontrol activity against *Armillaria mellea*, a destructive fungal pathogen responsible for root rot in multiple crops.

Methods Using a mass selection approach, we identified 155 bacterial isolates with antifungal activity, which we further screened to select four *Pseudomonas* spp. isolates that exhibited strong in vitro inhibitory effects on *A. mellea*. Whole-genome sequencing of these isolates revealed biosynthetic gene clusters encoding antifungal metabolites and siderophores that are potentially involved in pathogen suppression and plant growth promotion. We further assessed their effect on plant microbiomes and growth performance in a microcosm experiment with olive plants (*Olea europaea*).

Results Our results show that inoculation with live bacterial isolates, compared to inactivated isolates, increased plant biomass while exerting limited effects on native root and rhizosphere microbiomes. Additionally, we observed indirect effects on plant biomass through plant-associated microbial communities, suggesting a complex interplay between the host and bacterial isolates in the rhizosphere, with an eventual impact on plant health.

Conclusions Soil-derived *Pseudomonas* spp. can be effective biocontrol agents against *A. mellea*, and their ability to enhance plant growth while maintaining microbiome stability makes them promising candidates for field application, promoting microbiome-based solutions in modern agriculture.

Keywords Armillaria root rot · Soilborne pathogen · Metabarcoding · Olive

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M. M. Aci · G. E. Agosteo · G. Pelle · A. Malacrinò · L. Schena
Department of Agriculture, Università degli Studi Mediterranea di Reggio Calabria, Reggio Calabria, Italy

A. Malacrinò (✉)
Department of Biological Sciences, Clemson University, Clemson, SC, USA
e-mail: antonino.malacrino@gmail.com; amalacr@clemson.edu

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Introduction

Crop protection against plant pathogens is becoming increasingly challenging as a consequence of agricultural intensification, global changes, increasing resistance to fungicides, and overall reduction in barriers to pathogen spread (Singh et al. 2023). While chemical solutions are not sustainable in the long term, microbial communities thriving in soils and plants have demonstrated high potential for use in guaranteeing plant health (Banerjee and van der Heijden 2023; Beattie et al. 2024). For example, targeted manipulation of the soil microbiome was able to suppress the pathogens *Ralstonia solanacearum* and *Fusarium oxysporum* in tomato monocultures (Deng et al. 2021). Interestingly, Yang et al. (2023) found that rhizosphere phage communities were involved in the suppression of *R. solanacearum*, a role previously attributed mostly to prokaryotes. Managing the soil microbiome is a promising strategy for biotic and abiotic stress management (Compant et al. 2025), however we are still in the process of understanding how soil microbiome composition can be steered to achieve specific outcomes, including crop protection.

Soil microbiome composition and function are key to ensuring plant health (Banerjee and van der Heijden 2023), and there is evidence suggesting that variation in the soil microbiome composition can have both beneficial and detrimental effects on plant health and growth. For example, Gu et al. (2022) found that variation in rhizosphere microbiome composition during early plant development can lead to different susceptibility to the pathogen *R. solanacearum*. Similarly, variations in the soil microbiome can predict the crop growth response to arbuscular mycorrhizal fungi (Lutz et al. 2023). While we continue to investigate the effects of soil microbiome variability on plant traits, there are other possible ways to harness the soil microbiome to protect crops against biotic stressors, as soil microbiome composition is a major determinant in facilitating or contrasting plant pathogens (Bollmann-Giolai et al. 2022; Li et al. 2021a; Malacrinò et al. 2022; Yu et al. 2022).

The use of microorganisms to contrast plant diseases has been pioneered long time ago, however, biocontrol programs using individual microbial isolates have often led to inconsistent results depending on the receiving environment (Compant et al. 2025). Instead of focusing on a one-size-fits-all solution, with a single microorganism or microbial consortium to be used in a wide variety of environmental contexts, we can begin to explore the local soil biodiversity to select potential biocontrol agents that are adapted to local conditions. This represents an opportunity to identify microorganisms that can aid plant protection and growth without the use of synthetic chemicals. The use of microbial antagonists is particularly interesting in the context of contrasting soil-borne plant pathogens, which are particularly difficult to control with synthetic chemicals. Indeed, biocontrol agents can thrive in soil and exert their effects on plant pathogens by antibiosis, competition for space and nutrients, and production of antifungal secondary metabolites (Huang et al. 2018; Jiang et al. 2018; Liu et al. 2018; Yue et al. 2023). In addition to direct pathogen suppression, biocontrol agents can enhance plant growth, induce systemic resistance, and promote the assembly of healthy plant microbiomes (Dudenhöffer et al. 2016; Tahir et al. 2017; Zhang et al. 2019a). Biocontrol agents can also work in concert with other beneficial microbial taxa, contributing to the suppression of soil-borne pathogens such as *R. solanacearum*, *F. oxysporum* (Cao et al. 2018; Hu et al. 2021), *Phytophthora capsici* (Sang et al. 2018; Zhang et al. 2019b), *Botrytis cinerea* (Jiang et al. 2018) and *Rhizoctonia solani* (Moshe et al. 2024). Although the use of microbial biocontrol agents seems promising, there is limited research on the effects of their introduction on the plant microbiome (Araujo et al. 2019; Hu et al. 2021; Huang et al. 2021; Moshe et al. 2024; Yaghoubi Khanghahi et al. 2022), which is a crucial first step in understanding their impact on the wider ecological community (Li et al. 2022).

In this study, we isolated potential microbial biocontrol agents from the soil microbiome, tested their in vitro efficacy against a soil-borne fungal plant pathogen, and assessed their impact on the plant root and rhizosphere microbiomes. We hypothesized that using a similar approach to that used in our previous study (Librizzi et al. 2022) we would be able to select a panel of microorganisms from soils with potential biocontrol activity against soil-borne fungal pathogens. We also hypothesized that the potential biocontrol agents we isolated are adapted to the local environmental conditions, and thus we predicted that their overall impact on the belowground soil microbiome might be limited, while maintaining their

positive effects on plants. In this study we focused on *Armillaria mellea*, the agent of the Armillaria root rot (Baumgartner et al. 2011). This pathogen colonizes and degrades root systems, causing root rot and eventual tree death (Baumgartner et al. 2011) in a wide range of host plants, including fruit and nut crops, forest trees, and urban trees worldwide (Devkota and Hammerschmidt 2020; Kim et al. 2022). Current management strategies for this pathogen heavily rely on practices that are often labor-intensive, detrimental to the environment, and of limited efficacy (Kim et al. 2022). We selected this organism because it is a virulent soil-borne fungal plant pathogen, which causes severe losses in both forest and agricultural settings (Kim et al. 2022), and harnessing the soil microbiome could be a key strategy for managing this pathogen.

Materials and methods

Selection of potential biocontrol agents

In our previous study (Librizzi et al. 2022) we successfully isolated bacteria with antifungal activity from soil. In this study, we used the same approach to select potential biocontrol agents within the culturable portion of the soil microbiome. While the focus of this study is *A. mellea*, the way this fungus grows in vitro (Fig. S1) makes it difficult to implement our previous protocol. Thus, the mass selection of potential biocontrol agents was performed using *Rosellinia necatrix*. We selected this organism because it grows uniformly on PDA and its strategy as a plant pathogen infecting a wide range of hosts (e.g., apple, apricot, and avocado trees) is very similar to *A. mellea*, allowing us to easily screen for bacteria with high biocontrol potential against *A. mellea* as well. Briefly, we co-inoculated PDA plates with (i) a microbial wash obtained from soils (bulk soil from top 20 cm) sourced from soils ranging cultivated and uncultivated areas (Table S1), and (ii) a mycelium suspension of *R. necatrix*. The mycelium suspension was obtained by culturing *R. necatrix* on PDA at 23 °C in the dark for ~1 week, collecting the mycelium in 5 mL tubes containing sterile PBS, and homogenizing it using a bead-mill homogenizer (30 Hz for 40 s) together with a mix of 425–600 µm glass beads. Petri dishes (diameter 90 mm) with PDA medium were then inoculated with 100 µL of soil microbial suspension and 100 µL of *R. necatrix* mycelium suspension, spread evenly with a sterile spatula, and incubated at 25 °C for 7 days. After incubation, we first selected 155 bacterial colonies showing a fungal inhibition halo. From this set, we then further selected 25 isolates among those with larger inhibition halo, trying to evenly subsample across different sampling locations and trying to diversify among size, color, and morphology of the colonies (Table S1).

Since the mass selection was performed on a different model species, we then tested the in vitro antifungal activity of the 25 isolates against *A. mellea* using dual-culture assays as in Librizzi et al. (2022) with minor modifications. Briefly, PDA Petri dishes were inoculated with each bacterial isolate by striking it with a spatula across the dish diameter (Fig. S1), and then *A. mellea* was inoculated on one of the two halves by placing a ~0.16 cm^2 agar plug from the pure culture. Each bacterial isolate was tested in triplicate, and a set of control plates ($n = 3$) inoculated with *A. mellea* only served as a control. Plates were then incubated at 24 °C in the dark and monitored for 4 weeks. The growth area of *A. mellea* in each plate was estimated from high-resolution images using the software ImageJ (Schneider et al. 2012). All data analyses were performed in R v4.4.0 (R Core Team, 2023). The growth area for each isolate and control group was fitted to a linear model using the package *lme4* v1.1 (Bates et al. 2015) setting the isolate identity as a fixed factor. Pairwise post-hoc contrasts were then estimated using the package *emmeans* v1.10.7 (Lenth 2017).

The results from the previous test (see below and Table S2 Supplementary Materials) helped us to select 4 bacterial isolates with in vitro antifungal activity against *A. mellea* (B11, B12, B13, and B17). To increase our confidence in their potential biocontrol activity, we performed an additional in vitro experiment in which each bacterial culture ($OD_{600} = 0.1$, corresponding to a concentration of ~10^8 CFU/mL) was evenly spread on PDA plates, and an agar plug (~0.15 cm^2) with mycelium of *A. mellea* was placed at the center of the plate. Plates (3 replicates per bacterial isolate) were incubated as described above and the growth area of *A. mellea* was measured as described above. The surface area from each isolate and control group was fitted to a linear model using the package *lme4* setting the isolate identity as

a fixed factor. Pairwise post-hoc contrasts were estimated using the package *emmeans*.

Bacterial whole genome sequencing

We sequenced the genome of each of the four isolates selected above to identify potential mechanisms through which they can help plants contrast *A. mellea* (e.g., production of antifungal compounds or promotion of plant growth) and attempt their taxonomic identification. Each isolate was grown in LB broth and incubated overnight at 37 °C with continuous shaking (180 rpm). High-molecular-weight DNA was extracted from bacterial cultures using the ISOLATE II Genomic DNA Kit (Bioline, USA), according to the manufacturer's instructions. DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, USA) and its integrity was assessed by gel electrophoresis (0.8% w/v agarose gel). Sequencing libraries were constructed using the Native Barcoding Kit 96 V 14 (Oxford Nanopore Technologies, UK) and sequenced on a GridION instrument equipped with a FLO-MIN-114 R10.4.1 flow cell (Oxford Nanopore Technologies, UK). Raw data were processed using the Nextflow (Di Tommaso et al. 2017) pipeline wf-bacterial-genomes (https://github.com/epi2me-labs/wf-bacterial-genomes). Briefly, genomes were assembled using Flye v2.9.2 (Kolmogorov et al. 2019) and polished using medaka 1.11.3 (https://github.com/nanoporetech/medaka). Genome assembly quality was assessed using the KBase online platform (Arkin et al. 2018), CheckM v.1.0.18 (Parks et al. 2015) and Quast v.4.4 (Mikheenko et al. 2016). No contamination was found. The identity of our isolates was tentatively assigned using TYSG (Meier-Kolthoff et al. 2022) and the Average Nucleotide Identity (ANI) (Goris et al. 2007) was calculated using FastANI v1.3.3 (Jain et al. 2018). Genomes were annotated using the Prokka v1.14.6 (Seemann 2014). Secondary metabolite biosynthetic clusters were identified using antiSMASH v7.0 (Blin et al. 2023).

Microcosm experiment

We tested the influence of each of the four selected microbial isolates (B11, B12, B13, and B17; see Results below) on plant growth and on the root and rhizosphere microbiome in a full-factorial microcosm experiment that included plants inoculated with each individual microbial isolate ($n = 4$) which was either alive (active) or inactivated (control) by autoclavation (121 °C for 15 min) ($n = 2$), and each combination was replicated 10 times, for a total of 80 plants. We selected inactivated bacterial cells as control to account for the potential influence of other components of the inoculum (e.g., metabolites, nutrients), other than live cells, in explaining our results. One-year-old olive plants (cultivar Leccino) were sourced from a commercial nursery located in Gioia Tauro (Italy). After a two-week adaptation to our greenhouse conditions, plants were transferred to 1.5 L pots filled with a mix of field soil and sand (1:1 volume). Plants were then watered, and inoculated after 48 h. Bacterial isolates were grown in LB broth for 24 h at 28 °C in a rotary shaker (180 rpm), harvested by centrifugation at 5,000 rpm for 15 min, and resuspended in sterile PBS (1 ×). The concentration of the bacterial suspension was adjusted to approximately 10^8 CFU/mL ($OD_{600} = 0.1$). Olive plants were inoculated with either 50 mL of live bacterial suspension or 50 mL of autoclaved bacterial suspension according to the treatment group. Plants were then fully randomized and watered three times per week. After four months, we harvested the plants and collected samples to characterize the root and rhizosphere bacterial communities and to measure the above- and belowground biomass. Rhizosphere soil samples (~ 50 mg) were collected by removing the bulk soil loosely attached to the roots by shaking and collecting the soil particles firmly attached to the roots. Plants were then separated into shoots and roots. The roots were washed with tap water, surface-sterilized by dipping in 2% sodium hypochlorite for 30 s, rinsed twice with sterile distilled water, and dried with paper towel. A portion of the root sample (~ 50 mg) was collected for metabarcoding analyses. Both root and rhizosphere samples for metabarcoding analyses were then stored at −80 °C, while the rest of the plant material was oven-dried at 60 °C for one week before being weighed. We tested the influence of the bacterial isolate and its status (active, inactive) on dry biomass by fitting two separate linear models (one for shoot and one for root biomass) using the package *lme4*, using isolate status (active, inactive), identity (B11, B12, B13, and B17), and their interaction as fixed factors. Post-hoc contrasts between groups were estimated using the package *emmeans*.

Metabarcoding

DNA extraction and library preparation from the rhizosphere soil and roots samples (80 plants, 160 samples) were performed as reported in Malacrinò et al. (2021). Briefly, samples were lyophilized and powdered, and the DNA was extracted from ~25 mg of sample using a phenol−chloroform protocol. After quality control, libraries were prepared targeting the V4 region of the bacterial 16S rRNA gene using the primers 515f and 806r (Caporaso et al. 2012). PCRs were also performed on i) non-template controls in which DNA was extracted from nuclease-free water to account for possible contamination during the DNA extraction process, and ii) negative controls in which template DNA volume was replaced with molecular-grade water. The samples were then purified using an Agencourt AMPure XP kit (Beckman Coulter, USA) and subjected to a second short-run PCR to integrate Illumina adaptors. After a second purification, libraries were quantified using a Qubit fluorometer (Thermo Fisher Scientific Inc., USA), normalized using nuclease-free water, pooled at an equimolar ratio, checked for the correct size on a TapeStation 4150 instrument (Agilent, USA), and sequenced on an Illumina MiSeq instrument (Illumina, USA) on a 300PE flow cell according to the manufacturer's instructions.

After quality check and adapter trimming using TrimGalore (Krueger et al. 2023), the paired-end reads from Illumina sequencing were processed in R v4.4.0 (R Core Team, 2023) using the DADA2 pipeline v1.22 (Callahan et al. 2016) to identify the ASVs and remove low-quality data and chimeras. ASV sequences were taxonomically annotated using the SILVA database v138 (Quast et al. 2013), aligned using MAFFT v7.525 (Katoh and Standley 2013), and a phylogenetic tree was built using FastTree v2.1.10 (Price et al. 2009). The ASV table, taxonomic information, metadata, and phylogenetic tree were combined into a single object using the package *phyloseq* v1.48 (McMurdie and Holmes 2013). Before analyses, we removed the mitochondrial/plastidial reads from the dataset, potential contaminants identified from non-template and negative controls using the package *decontam* v1.24 (Davis et al. 2018), and singletons. Data were also normalized using the package *wrench* v1.22 (Kumar et al. 2018) before any analysis requiring the estimation of taxa relative abundances. Samples with less than 1,000 reads were eliminated from the downstream analyses. After the quality check, the dataset included 120 samples with at least five replicates per group (Table S3).

Bacterial community diversity was estimated by calculating Faith's phylogenetic diversity index using the package *picante* v1.8.2 (Kembel et al. 2010). The effect of isolate identity and status on bacterial microbiota diversity was tested by fitting a linear model using the package *lme4* v1.1 (Bates et al. 2015), where isolate status (active, inactive), identity (B11, B12, B13, and B17), and their interactions were set as fixed factors, and pairwise contrasts (FDR-corrected) were estimated using *emmeans* package v1.10.7. The unweighted UniFrac distance between pairs of samples was calculated using *vegan* package v2.6 (Dixon, 2003) and visualized using Non-metric Multi Dimensional Scaling (NMDS). Differences in the compositional structure of root and rhizosphere soil bacterial communities were tested using PERMANOVA (999 permutations) implemented in the *vegan* package (Dixon, 2003) considering isolate status (active, inactive), identity (B11, B12, B13, and B17), and their interaction as fixed factors. FDR-corrected post hoc contrasts were performed using the package *RVAideMemoire* v0.9 (Hervé, 2022). ASVs that were differentially abundant in inoculated plants were identified separately for each bacterial isolate and for the roots and rhizosphere soil using the *DESeq2* package v1.48 (Love et al. 2014).

A Structural Equation Model (SEM) was constructed using the package *piecewiseSEM* v2.3.0.1 (Lefcheck 2016) to test the influence of isolate status (active, inactive) on the microbial diversity (Faith's phylogenetic diversity) and composition (first NMDS component) of each compartment (roots and rhizosphere), and their direct and indirect influences on root and shoot biomass. Each model within the structural equation model was a linear-mixed effects model that included the isolate identity (B11, B12, B13, and B17) as a random effect.

Results

In the first step of this study, we mass selected 155 bacterial isolates with potential biocontrol activity against *A. mellea*, which were then further reduced to 25 isolates as described above. We tested these 25

isolates in dual culture assays against *A. mellea* and found that 13 of them significantly reduced fungal growth compared with the control (Table S2). Among these, strains B11, B13, and B17 were the most effective at reducing fungal growth (Table S2). Interestingly, isolate B12, which significantly reduced fungal growth, also completely inhibited the development of rhizomorphs in vitro, and it was thus deemed interesting for further assays, as rhizomorphs play a major role in the pathogenicity and spread of *A. mellea*.

Then, we performed additional dual culture assays to further confirm the in vitro antifungal activity of the 4 bacterial isolates (B11, B12, B13, and B17) we selected to perform the downstream experiments. We found that the presence of bacterial isolates significantly influenced fungal growth in vitro ($F_{4, 10} = 375.94$, $p < 0.001$), and isolates B11, B13, and B17 almost completely inhibited mycelial growth compared to the control (Fig. 1A-B, Table S4). Also in this test, isolate B12 prevented the development of rhizomorphs (Fig. 1A).

We performed whole genome sequencing to identify the bacterial strains by assembling their draft genomes and characterizing their genome content. The draft genomes were first compared towards *Pseudomonas* reference genomes using TYGS (Meier-Kolthoff et al. 2022) (Fig. S2) which allowed the tentative identification of two isolates, *Pseudomonas spelaei* (B12, ANI 98.05%) and *Pseudomonas paracarnis* (B13, ANI 99.06%) (Table S5). Strains B11 and B17 did not closely match any reference species within the genus *Pseudomonas*, with the highest ANI values toward *P. koreenensis* (B11, ANI 91.35%) and *P. uvaldensis* (B17, ANI 88.99%) (Table S5). Pairwise comparison between the draft genomes of the four isolates resulted in an average ANI of 83.41%. When focusing on the predicted bioactive compound biosynthesis gene clusters, we found that the genomes of the four isolates included clusters associated with the production of several molecules (Table S6), which included: i) lipopeptides, polyketide, nitrile, and pyrrole derivatives with potential antifungal activity (fengycin, fragin, hydrogen cyanide, lokisin, putisolvin, pyrrolnitrin, and viscosinamide A/pseudodesmin A); ii) siderophores with potential plant growth promotion (pyoverdine, EDHA, histicorrugatin, and pseudomonine); iii) the antibiotic lankacidin C; and iv) the pathogenicity factor syringomycin. Fengycin, pyoverdine, and lankacidin C clusters were found in all strains, whereas other clusters were found in only one or two strains (Table S6).

We then tested the impact of bacterial isolates on the diversity and structure of the bacterial microbiota in the plant roots and rhizosphere. After cleanup

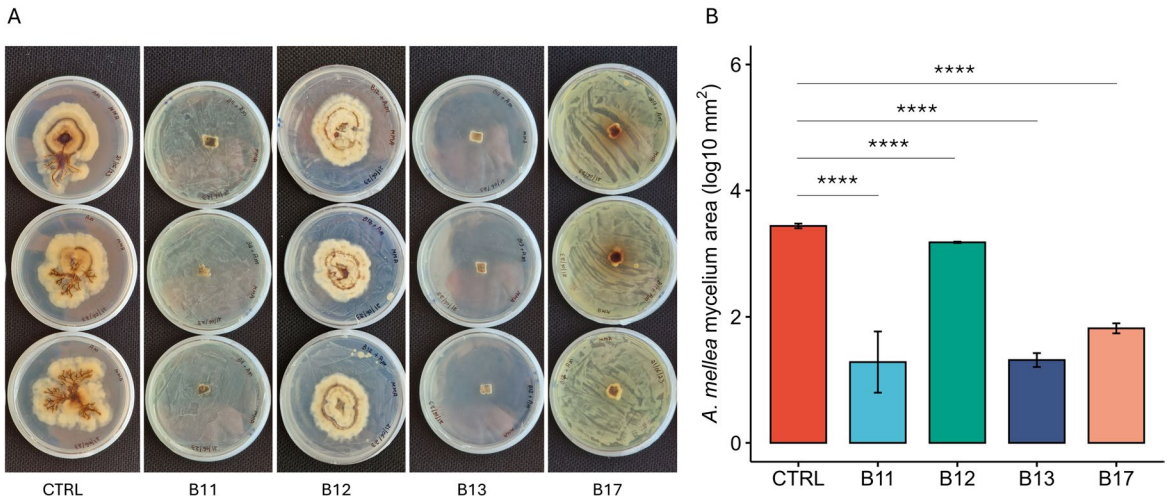

Fig. 1 In vitro antifungal activity of four bacterial isolates (B11, B12, B13, and B17). **A** Representative PDA plates of dual culture essay with *A. mellea* in pure culture (CTRL) or co-cultured with bacterial strains B11, B12, B13 and B17. **B** Efficacy of the four bacterial isolates in reducing the mycelium area (log10 transformed data). The pairwise comparison between each isolate and the control group (CTRL) are shown as asterisks on the top of each pair of bars ($p < 0.0001$; see Table S4 for full details)

and filtering, metabarcoding yielded 611,029 reads (on average ~6,500 reads/sample; min 1,096; max 22,892) and identified 4,193 ASVs. When looking at the variation in microbial phylogenetic diversity, we found a significant interaction between isolate status (active and inactive) and isolate identity (B11, B12, B13, and B17) in the roots ($F_{3, 46} = 4.59$, $p = 0.0067$; Table S7), and rhizosphere soil samples ($F_{3, 38} = 2.95$, $p = 0.045$; Table S7). The post-hoc contrasts suggested a higher bacterial diversity in the roots of plants inoculated with isolate B12 ($p = 0.012$) and in the rhizosphere of plants inoculated with isolate B17 ($p = 0.016$) (Fig. 2), compared to the respective inactivated inocula.

We also found a significant interaction between isolate status and identity on the structure of the root and rhizosphere bacterial communities (Table 1, Fig. 3). However, post-hoc pairwise comparisons (FDR-corrected) did not reveal any differences within all combinations of isolate status and identity ($p > 0.05$, Table S8), suggesting that the effects of these factors on the root and rhizosphere bacterial communities were marginal.

At the genus level, both the root and rhizosphere soil bacterial communities varied when inoculated with the active bacterial isolates (Fig. S3). When examining the ASVs that were differentially abundant between plants inoculated with the active and inactive inocula in rhizosphere soil and roots, we identified a total of 46 ASVs (Fig. 4; Table S9). In roots, ASVs identified as *Kutzneria* sp. and Sandaracinaceae (B11), *Actinoplanes* sp., *Pedomicrobium* sp., *Lacunispharera* sp. and other 9 taxa identified at the family level (B12) were more abundant in plants inoculated with the active strains. On the other hand, ASVs identified as *Vicinamibacter, Pir4 lineage, Ahniella*

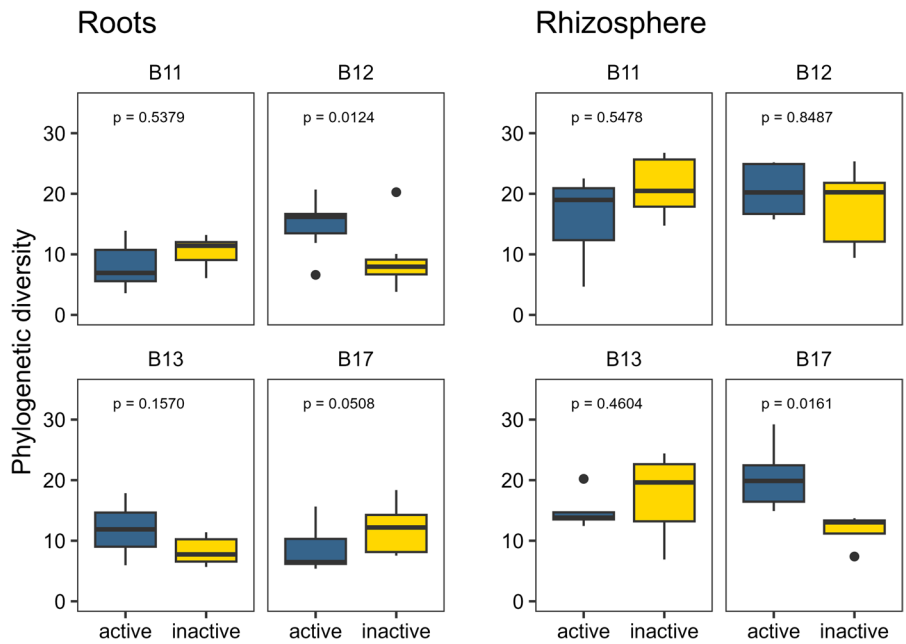

Fig. 2 Influence of isolate status (active, inactive) on the phylogenetic diversity of the root (left) and rhizosphere soil (right) bacterial communities of olive plants, for each isolate used as inoculum. Pairwise comparison p-values are FDR corrected

Table 1 Results from PERMANOVA testing the effect of isolate status (active, inactive), isolate identity (B11, B12, B13, B17), and their interactions on the structure of olive root and rhizosphere soil bacterial communities

Factors	df	Roots			Rhizosphere soil		
		R^2	F	p	R^2	F	p
Isolate status	1	0.013	0.71	0.876	0.024	1.12	0.208
Isolate identity	3	0.049	0.86	0.788	0.061	0.94	0.605
Isolate status × identity	3	0.083	1.46	**0.022**	0.103	1.58	**0.002**

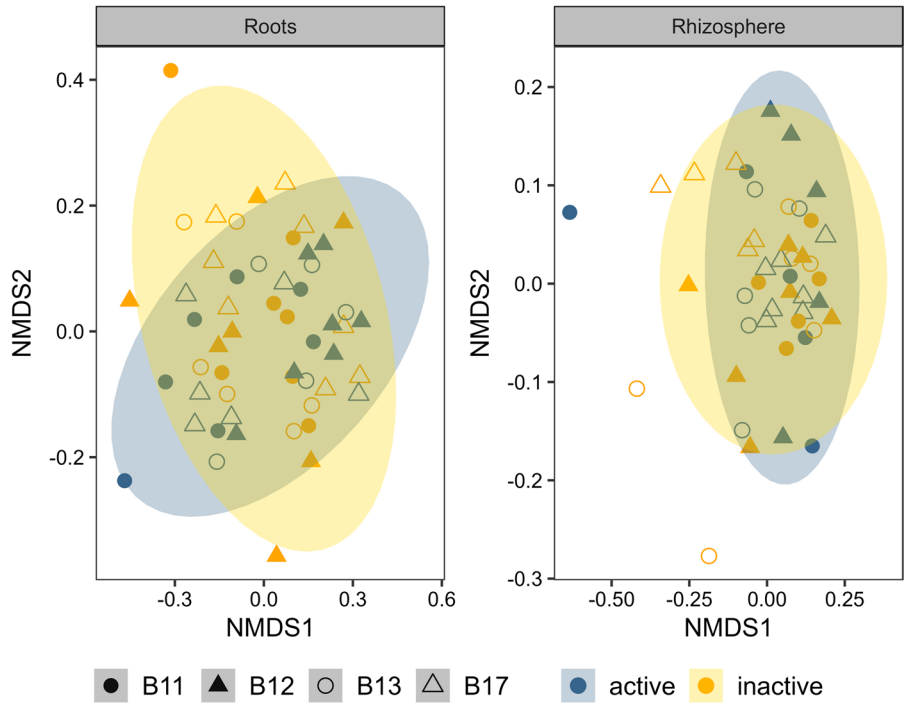

Fig. 3 Non-metric Multi-Dimensional Scaling (NMDS) plots built using an unweighted Unifrac distance matrix showing the influence of isolate status (active, inactive) and isolate identity (B11, B12, B13, and B17) on the structure of the bacterial communities of roots (left) and rhizosphere soil (right) (points and 95% CI ellipses are colored by isolate status)

sp. and Microbacteriaceae (B11), *Bacillus* sp. (B12), *Hydrogenophaga* sp., *Altererythrobacter* sp., *Actinoplanes* sp. and Xanthobacteraceae (B13) were more abundant in plants inoculated with inactive strains. No ASV was significantly influenced by inoculation with isolate B17 (Fig. 4; Table S9). In the rhizosphere, inoculation with active isolate B17 increased the abundance of 16 ASVs belonging to different genera, such as *Bradyrhizobium, Blaudia and Deviosa* (Fig. 4; Table S9). On the other hand, ASVs identified as *Pseudolabrys* sp. (B11), within the orders Polyangiales and Tepidisphaerales, the families Nitrosomonadaceae, Puniceicoccaceae, Fibrobacteraceae, and within the genus *Lacunisphaera* (B12) were more abundant when plants were inoculated with inactive strains (Fig. 4; Table S9). No ASV was significantly affected by the isolate B13.

We also tested whether our bacterial isolates influenced the plant dry biomass of roots and shoots and found that isolate status (active or inactive) was the main factor driving dry biomass changes in both roots ($F_{1, 52} = 53.45$; $p < 0.0001$) and shoots ($F_{1, 52} = 23.15$; $p < 0.0001$) (Table S10). The post-hoc contrasts revealed a significant increase in the biomass of all olive plants inoculated with active bacterial isolates compared to those treated with inactive bacterial isolates (Fig. 5), with the exception of the shoot biomass of plants inoculated with isolate B17.

The SEM approach confirmed that the inoculation of bacterial isolates directly influenced both shoot and root biomass, and showed a significant influence of bacterial isolates on the diversity and structure of both root and rhizosphere microbial communities (Fig. 6). The influence of bacterial isolate status on the bacterial microbiota structure and phylogenetic diversity of root bacterial communities in plants treated with active bacterial isolates was significantly associated with root biomass. Similarly, rhizosphere phylogenetic diversity also had a significant effect on root biomass (Fig. 6). Interestingly, an increase in root bacterial diversity was associated with lower root biomass, whereas a higher rhizosphere bacterial diversity was associated with higher root biomass (Fig. 6).

Discussion

In this study, we were able to select four *Pseudomonas* spp. isolates that showed strong in-vitro antifungal activity against the soil-borne fungal pathogen *A. mellea*. The genomes of these isolates

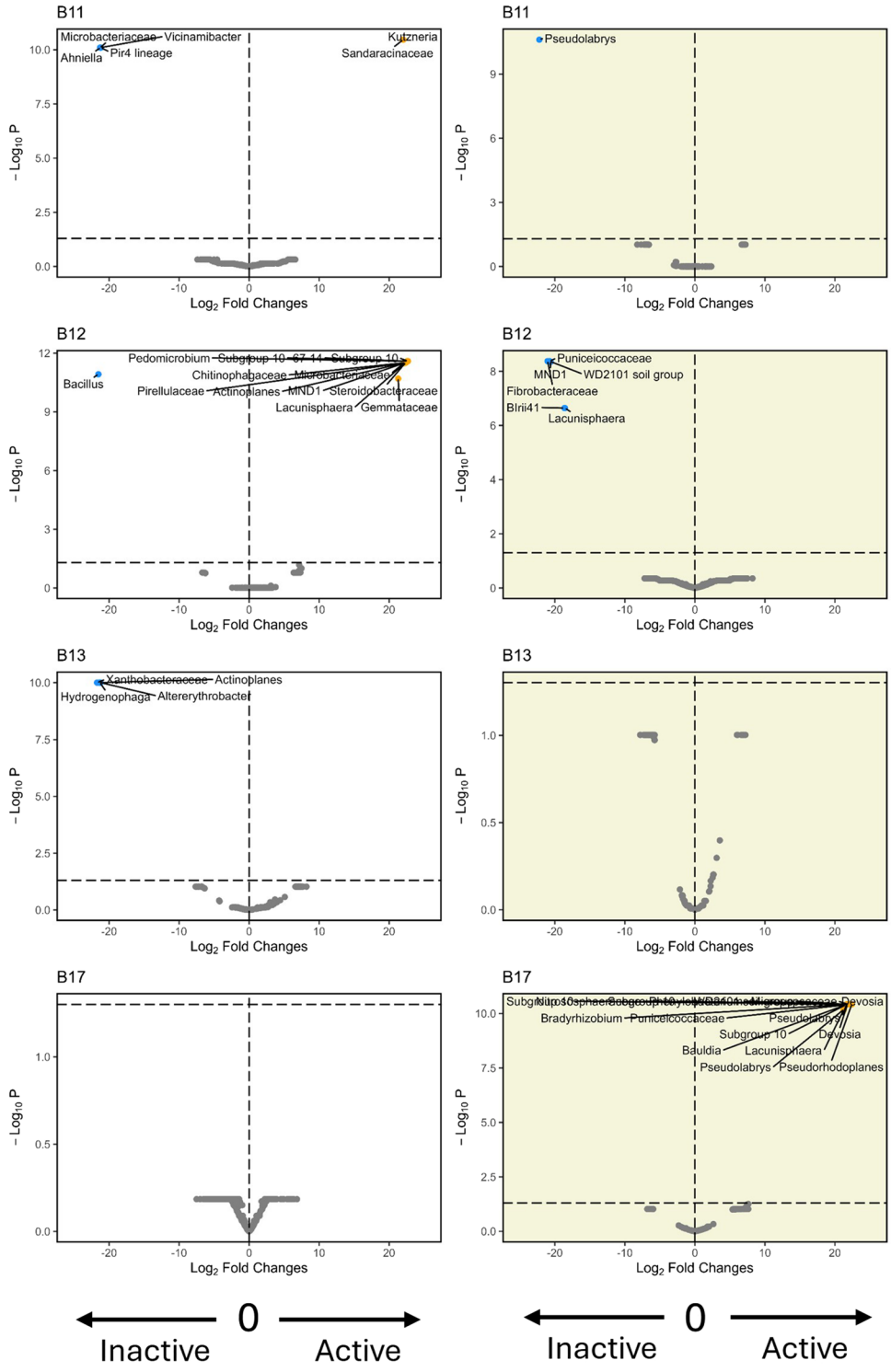

Fig. 4 Volcano plots showing the ASVs differentially abundant in active and inactive inocula in roots (left, white background) and rhizosphere soil (right, beige colored background) for each isolate. For more information about each ASV, see Table S9

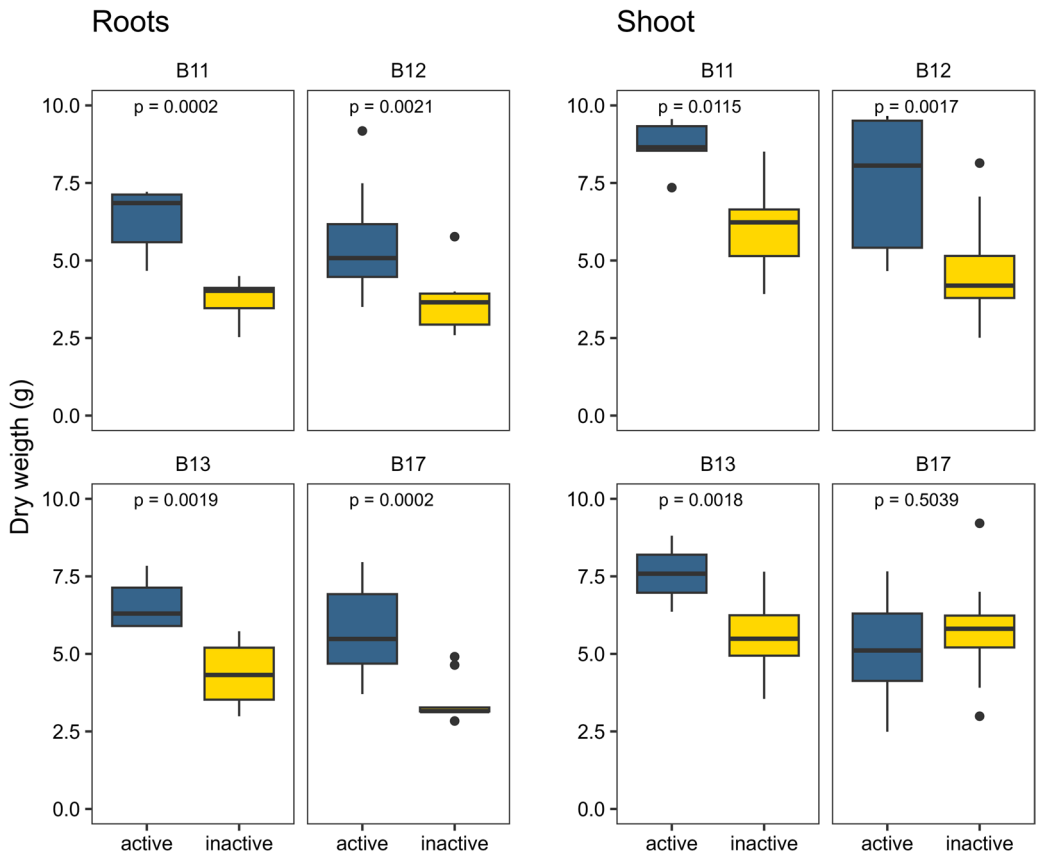

Fig. 5 Influence of bacterial isolate status on the dry biomass of roots (left) and shoot (right) of olive plants for each bacterial isolate, with FDR-corrected post-hoc p-values for each comparison between active and inactive isolates

contained gene clusters coding for secondary metabolites with antifungal and plant growth-promoting activity. We also tested the influence of these bacterial isolates on the plant belowground microbiome (roots and rhizosphere soil) and found little overall change as a consequence of bacterial inoculation. We also found that our bacterial isolates had both direct and microbiome-mediated effects on plant shoot and root biomass.

Using the selection process described in our previous study (Librizzi et al. 2022) we were able to select four bacterial isolates with strong in vitro inhibition against *A. mellea*. Through genome sequencing, we were able to identify their taxonomic identity and their genome content. All isolates were identified as belonging to the genus *Pseudomonas*. Two isolates, B12 and B13, were identified as *P. spelaei* and *P. paracarnis* when compared to the reference genomes. The other two isolates were not identified at the species level. Although the taxonomic classification of isolates B11 and B17 is not fully accurate, *Pseudomonas koreensis* (tentative identification for isolate B11) was first described as a new species by Kwon et al. (2003), and has been reported as a plant growth-promoting bacterium and antagonist of plant pathogenic fungi (Gu et al. 2020). *Pseudomonas spelaei* (isolate B12), *P. paracarnis* (isolate B13), and *P. uvaldensis* (tentative identification of isolate B17) are newly described species (Khanal et al. 2022; Lick et al. 2021; Švec et al. 2020) and to the best of our knowledge, they have not been reported as biocontrol agents. While *P. uvaldensis* has been reported as a plant pathogen in a previous study (Khanal et al. 2022), it is important to note that this is a tentative identification, and that the boundaries between pathogenic and beneficial *Pseudomonas* within the plant microbiome are not always well defined (Li et al. 2021b). It is not surprising that all identified

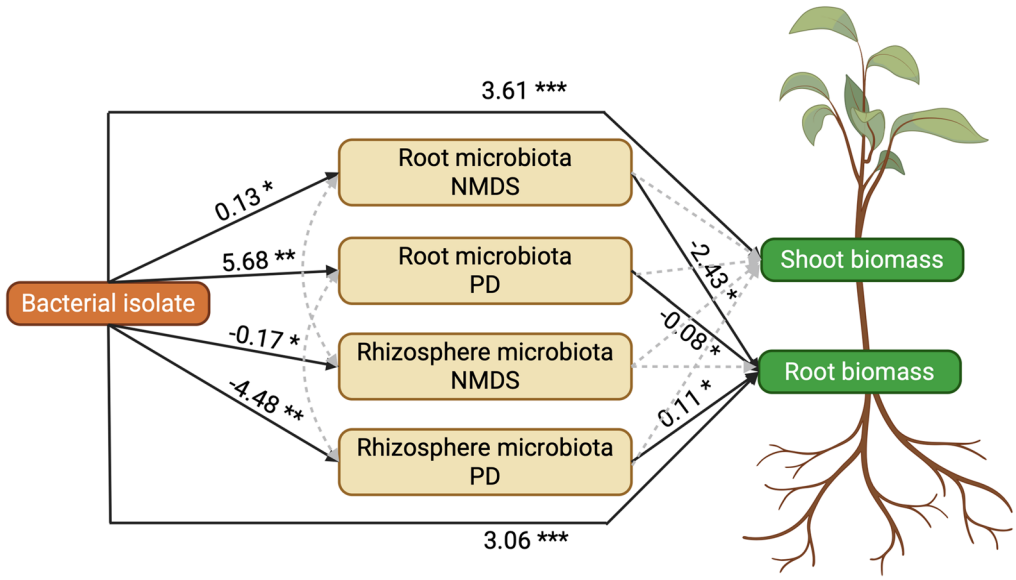

Fig. 6 Piecewise Structural Equation Model highlighting the significant impact of treatments with active bacterial isolates on the structure (first NMDS dimension) and the phylogenetic diversity (PD) of the bacterial communities and roots and shoots biomass of the olive plants. Solid lines represent significant relationships with estimates value and significance (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). Dashed lines represent relationships with non-significant effects. Created in BioRender

biocontrol agents belong to the genus *Pseudomonas*, as several previous studies have shown their ability to suppress plant disease agents (Chetan et al. 2024; Dimaria et al. 2024; Dimkić et al. 2022). These *Pseudomonas* biocontrol agents can produce secondary metabolites that inhibit fungal growth, promote plant growth, and induce plant systemic resistance (Dimkić et al. 2022).

When testing each bacterial isolate against *A. mellea* using in vitro dual-culture assays, our bacterial isolates exhibited antifungal activity. Strains B11, B13, and B17 almost completely prevented the growth of *A. mellea*, and the fungal growth inhibition area suggested the production of antifungal compounds. On the other hand, isolate B12, while reducing the growth of the mycelium, also prevented the development of rhizomorphs. While several mechanisms can be responsible for this effect (e.g., competition for spaces and resources), this isolate has potential use as a post-infection treatment because of its effect on rhizomorphs, the main strategy *A. mellea* uses to spread between hosts (Devkota and Hammerschmidt 2020). When we investigated the genome content of our isolates, we found several genes associated with the production of molecules with antifungal activity (fengycin, fragin, hydrogen cyanide, lokisin, putisolvin, pyrrolnitrin, and viscosinamide A/pseudodesmin A) and siderophores (pyoverdine, EDHA, histicorrugatin, and pseudomonine). Among the detected clusters, fengycin, pyrrolnitrin, and hydrogen cyanide have been previously shown to inhibit soilborne pathogens (Blin et al. 2023). For instance, the detection of pyrrolnitrin in B17 and putisolvin in B12 suggests that these isolates may interfere with fungal cell membranes and biofilm formation (Blin et al. 2023; Liu et al. 2018). Also, the presence of siderophore-producing gene clusters, such as pyoverdine, EDHA, histicorrugatin, and pseudomonine, indicates the ability of these isolates to sequester iron, thereby limiting the availability of this critical nutrient to fungal pathogens (Glick 2012). The different panel of bioactive compounds associated with each tested antagonistic bacteria aligns with previous findings showing that different biocontrol agents deploy diverse strategies to suppress fungal pathogens (Huang et al. 2018; Jiang et al. 2018) and suggests their potential combination in consortia to increase their efficacy and reliability in contrasting *A. mellea* under field conditions.

Although the effects on plant biomass showed that our bacterial isolates had a direct effect on plant traits, it is also possible that their activity was mediated by interactions with other microorganisms. For example, the microbiome of plants inoculated with isolates B11 and B17 was enriched with specific bacterial groups, for example *Kutzneria* and *Bradyrhizobium*, which are known for their plant growth-promoting properties (Devi et al. 2021; Gomez et al. 2023). Therefore, our bacterial isolates may have worked alongside other resident members of the microbiome to influence plant growth. These effects, along with the potential induction of systemic plant resistance, highlight the importance of harnessing soil microbiome to improve plant health for sustainable agriculture. The results from the microcosm experiment suggest that inoculation with our bacterial isolates had a limited impact on the plant root and rhizosphere microbiome. At the same time, bacterial isolates influenced plant biomass both directly and indirectly through variation in plant microbiome diversity and structure. Previous studies also found that variation in the belowground plant microbiome can lead to variation in plant traits (Van Nuland et al. 2021), and even effects on aboveground pathogens and herbivores (Deng et al. 2021; Malacrinò and Bennett 2024). In this case we also observed that little variation in the plant microbiome can have tangible functional consequences for plants, similarly to what has been found in other models (Gu et al. 2022). However, we expected to observe an enrichment of *Pseudomonas* ASVs in plants exposed to the active inoculum. The fact that we did not observe this enrichment can be explained in two different ways, one technical and the other biological. Technically, we used metabarcoding to characterize the plant-associated microbial communities, which uses a small portion of the 16S rRNA to profile the taxonomical structure of entire microbial communities. While this is a powerful tool, it also has downsides and considering that *Pseudomonas* are widely abundant within plant microbiomes, the isolates we inoculated might not be differentiated from other *Pseudomonas* using this technique, and their abundance might have been confounded with other ASVs, decreasing the statistical power to observe them in the differential abundance analysis. Biologically, it is also possible that the strains we inoculated colonized our plants, as we observed the effects of the inoculation of live material compared to the inactivated inoculum, but at a low population size, enough to produce a biological consequence for plants, but not enough to detect differences using our tools. This supports the idea that it is indeed time to move from metabarcoding to shotgun metagenomics and metatrascriptomics, which helps in identifying members of microbiomes to the strain level and inferring their functional role within the community.

Our study lacks a direct test of the in vivo biocontrol activity of our bacterial isolates. All our data strongly suggest that their in vitro efficacy, combined with their in vivo plant growth promoting effects, can lead to promising results in microcosms and in the field. However, moving to field conditions requires a wide set of tests, which include different strains of the pathogen, different plant genotypes, and different starting soil microbial communities, which require a wider experiment that is outside the scope of this first study. In addition, *A. mellea* attacks woody species, and obtaining woody plants with a simplified microbial community, in order to control for the effects of microorganisms already associated with plants and that might confound our results, is particularly logistically challenging. Nevertheless, the selection of these bacterial isolates is an important first step in the investigation of the mechanisms underlying plant-microbiome-pathogen interactions that can be exploited to protect crops from disease agents.

We showed that selection from the soil microbiome using in vitro mass screening can help isolate bacteria with a high potential as biocontrol agents. Our bacterial isolates showed strong in vitro growth inhibition of the fungal plant pathogen *A. mellea*. The same isolates were also able to promote plant growth in a microcosm experiment with minimal impact on the plant microbiome. Thus, these bacterial isolates have a high potential for further study and use in the field, both as individual strains or as microbial consortia, to contrast the spread of *A. mellea* and, possibly, other fungal pathogens. While further analyses need to be performed to assess their influence on the fungal community, and beneficial fungi in particular, this study fully supports the idea that harnessing the soil microbiome to promote plant health is possible and can be tailored to specific environmental contexts, so that the potential biocontrol agents are already adapted to a certain environment, reducing the risks of their failure under field conditions. This fits within the wider context of microbiome

management, a powerful strategy that can promote the sustainability of agricultural systems without negatively influencing crop yield, thereby safeguarding both food safety and security.

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Data availability Raw metabarcoding data are available at the NCBI SRA under the Bioproject PRJNA1256103. Draft bacterial genomes are available as supplementary material.

Declarations

Competing interests All authors declare no financial or non-financial competing interests.

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