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Efficacy of new biocontrol agents against *Rhizoctonia solani* in lettuce evaluated through an automated imaging system and their effect on bacterial soil communities

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Abstract In recent years, biocontrol agents (BCAs) have gained attention as sustainable alternatives or complements to synthetic chemicals for managing phytopathogens and enhancing plant growth. In this study, six bacterial strains were evaluated as BCAs against *Rhizoctonia solani* (RS), a soil-borne pathogen affecting economically important crops. Two strains (*Bacillus* sp. B04A33 and *Psychrobacillus* sp. B04A42) were isolated from maize embryos, while *Lactiplantibacillus plantarum* strains (ITEM 17215, ITEM 17218, ITEM 18335 and S61) originated from food or animal sources. A commercial *Trichoderma*-based product (REMEDIER®, Gowan Italia) served as the control. Biocontrol effectiveness was assessed both *in vitro* and *in vivo* on lettuce using an automated imaging system for plant growth monitoring. In soils infected by *R. solani,* the inoculum of *s*trains B04A33, B04A42, and S61 enhanced germination and seedling vigor, and increased dry weight of shoots and roots. Root system development, analyzed via image processing in Matlab, confirmed improved growth. The qPCR analysis of soil samples showed that microbial abundance increased in non-treated (NT) and S61 + RS treatments, while *R. solani* levels decreased in B04A33 + RS and B04A42 + RS. Oxford Nanopore MinION sequencing of soil samples revealed dominance of *Pseudomonadota*, *Actinomycetota*, and *Bacillota*, with enrichment of Bacillaceae and Lactobacillaceae in B04A42 and S61 treatments, confirming the successful establishment of the inoculated strains within the soil microbiome. These results support both the potential of selected bacterial strains as effective BCAs against RS and the use of automated imaging in BCAs screening procedures, promoting sustainable and technology-assisted crop management.

Keywords *Lactiplantibacillus plantarum* · *Bacillus* · *Psychrobacillus* · Nanopore sequencing · Microbiome

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Introduction

Global food production must increase by ~ 70% by 2050 to meet growing demand (FAO 2009), yet agriculture faces resource depletion, climate change, and increasingly aggressive plant pathogens (Roth and Galyon 2024). Plant diseases account for 20–40% of annual global crop losses (Savary et al. 2019), with soil-borne fungi particularly problematic due to their persistence (Xia et al. 2024). Among them, *Rhizoctonia solani* Kühn (1858) is a highly destructive pathogen causing bottom rot and damping-off in lettuce, with yield losses up to 70% (Blancard et al. 2006). Its capacity to survive as sclerotia makes management difficult. Although soil fumigation and synthetic fungicides remain standard control measures (Fetyan et al. 2024), their extensive use has led to resistant strains (Cheng et al. 2020), disruption of beneficial soil microbiota (Wang et al. 2025), and environmental and food safety concerns (Beyuo et al. 2024). Increasing regulatory restrictions in Europe further highlight the need for sustainable alternatives (Mondal et al. 2017), particularly for short-cycle crops such as baby leaf lettuce.

Microbial biocontrol agents (BCAs) offer a promising approach through mechanisms including nutrient competition, antimicrobial metabolite production, lytic enzyme secretion, and induction of plant defenses (Passera et al. 2020). Well-studied genera include *Bacillus*, *Pseudomonas*, *Trichoderma*, and *Streptomyces* (Abbas et al. 2022; Serrão et al. 2024). More recently, lactic acid bacteria (LAB), particularly *Lactiplantibacillus plantarum*, have gained attention due to their GRAS and QPS status and their production of organic acids and antifungal compounds (Li et al. 2023).

This study evaluated plant growth-promoting traits and biocontrol potential of six bacterial strains against *R. solani*, focusing on soil bacterial community dynamics. Two strains (*Bacillus* and *Psychrobacillus* spp.) were isolated from maize embryos, and four *L. plantarum* strains from cereals, olives, and raw milk cheese. An automated, low-cost image-based system was used to assess plant growth, offering a faster, objective alternative to manual germination analysis (Colmer et al. 2020), and adaptable to greenhouse conditions.

Materials and methods

Microbial strains employed

Six bacterial isolates were used for *in vitro* and *in vivo* assays (Table 1). Four *Lactiplantibacillus plantarum* strains were included: ITEM 17215 (wheat bran), ITEM 17218 and ITEM 18335 (raw milk cheeses) from the Agri-Food Microbial Culture Collection (ITEM, CNR-ISPA, Bari, Italy), and S61 (fermented green olives; identified as in Abouloifa et al. 2020). Two additional isolates, B04A33 and B04A42, were obtained from maize embryos (Passera et al. 2021). Molecular identification (16S rRNA gene sequencing), preliminary biochemical characterization, antifungal activity, and effects on seed germination are reported in Supplementary Information (Sects. 1.1–1.4).

L. plantarum strains were cultured aerobically in MRS broth (Scharlab, Barcelona, Spain) at 30 °C for 24 h. B04A33 and B04A42 were grown on LB High Salt Agar (Difco Lab, Augsburg, Germany) at 22 °C for 48 h. All strains were maintained at 4 °C for short-term storage. *Rhizoctonia solani* strain RS1 (Anastomosis Group 4, AG-4), isolated from millet in 2012, was used as the target pathogen. Three additional fungal strains (Supplementary Information Sect. 1.1) were included where specified. Fungi were cultured on potato dextrose agar (PDA; Difco™) at 22 °C and stored at 4 °C.

In vivo antifungal assay against *Rhizoctonia solani* under greenhouse conditions

Greenhouse trials were conducted at 23 °C and 60% RH under a L:D 12:12 photoperiod, with supplementary dimmable (25%) LED lighting (Lumatek Zeus 600W Pro 2.9) positioned 1.3 m above the trays. *In vivo* antifungal activity was assessed on lettuce grown in substrate artificially infested with *Rhizoctonia solani* RS1 (AG-4). The inoculum was prepared as described by Vagher et al. (2014): three 1 cm^2 PDA plugs of actively growing mycelium were transferred to 300 g of hulled millet (autoclaved twice) and incubated for three weeks. The colonized substrate was dried at 30 °C for three days and incorporated into soil at 4 g kg^{-1} two weeks before sowing.

Bacterial suspensions (10^5 CFU ml^{-1} in Ringer's solution) were applied at 10 ml per 100 g

Table 1 Bacterial strains used in all experiments and their characterization

Strain	B04A33	B04A42	S61	ITEM 17215	ITEM 17218	ITEM 18335
Bacterial species	*Bacillus* sp.	*Psychrobacillus* sp.	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*
Isolation source	Maize embryo (Spinato di Gandino landrace)	Maize embryo (Spinato di Gandino landrace)	Fermented green olives	Raw milk cheese	Raw milk cheese	Wheat bran sourdough
Siderophore production	+	−	−	−	−	−
Phosphate solubilization	−	+	+	++	+	+
Catalase activity	+	+	−	−	−	−
Rhizoctonia solani RS1 GIP	44.4 ± 0.6^{b}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	n/a	n/a	n/a
Botrytis cinerea MG53 GIP	56.9 ± 5.9^{b}	35.5 ± 7.0^{a}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}
Fusarium verticillioides GV2245 GIP	100.0 ± 0.0^{d}	78.5 ± 2.7^{c}	66.4 ± 5.0^{b}	100.0 ± 0.0^{d}	100.0 ± 0.0^{d}	53.7 ± 8.6^{a}
Fusarium graminearum FG515 GIP	23.8 ± 21.7	12.8 ± 8.4	16.1 ± 0.7	16.9 ± 1.0	15 ± 1.1	18.1 ± 1.3

Table reports for each strain their name, species, isolation source, biochemical characteristics (siderophore production, phosphate solubilization and catalase activity) indicating the results with a graphical symbol (moderate detectable activity: "+"; strong detectable activity: "++"; no detectable activity: "–"), and results of *in vitro* biocontrol assays *versus* the indicated fungal strains reported as Growth Inhibition Percentage (GIP). GIP is expressed as a percentage value (%) ± SD, calculated as the mean of three replicates. Kruskal-Wallis non-parametric test, followed by Dunn's test, was used for statistical analysis ($p < 0.05$) and different letters indicate statistically significant differences among the effectiveness of bacterial strains on each fungi. No differences among different bacterial treatments were found on the GIP (%) of *F. graminearum.* Data are not available ("n/a") for some strains against *R. solani*

of infested soil, one week before and at sowing. Treatments were: B04A33 + RS, B04A42 + RS, S61 + RS, ITEM17215 + RS, ITEM18335 + RS, and ITEM17218 + RS (the last three included only in Trial 1). A commercial *Trichoderma*-based product (REMEDIER®, Gowan Italia; *Trichoderma gamsii* ICC 080 2% and *T. harzianum* ICC 012 2%) was applied at 250 g m^{-3} soil as reference (Trichoderma + RS). Controls were: RS (infested soil + Ringer's solution) and NT (non-infested soil + Ringer's solution).

Each treatment included four replicates (16 × 16 cm pots containing 100 g peat-based substrate, Vigorplant Semina BR-Balance) sown with 49 lettuce seeds. Trials lasted ten days and were repeated three times. Germinated seedlings were counted per pot to calculate germination rate. In Trials 2 and 3, ten seedlings per replicate were randomly collected, washed, separated at the root collar, dried at 65 °C to constant weight, and shoot (epigeal) and root (hypogeal) dry weights were recorded using a precision balance.

Image-based assessment of plant growth (and morphological responses) to bacterial treatments and *R. solani* inoculum

During the second and third *in vivo* experiments, epigeal responses were automatically monitored using the method by Torrente et al. (2025), with RGB cameras (Tapo C310) placed 48 cm above the pots. Each camera captured images of four replicates six times per day over ten days. Images were corrected for distortion and calibrated using AprilTag markers. Analysis focused on two goals: estimating seedling vigor via segmented green area (Excess Green Index) and counting germinated seeds by identifying new polygonal regions not present in earlier images. At the end of the trials, 49 plants per treatment were uprooted and washed to assess plant structure by photographing

leaves and roots on a blue background. Leaf area was segmented and measured, and the junction between shoot and root was identified using MATLAB's regionprops function. Primary root length was calculated by skeletonizing the root mask and measuring the distance from the junction to the farthest root apex. Root segmented area (mm^2), as the total area of the root system automatically detected in the image after segmentation, was also measured. Image-based results for germination and leaf area were compared with manual seed counts and aerial dry weight.

Soil analysis

DNA was extracted from three replicates of soil samples following the protocol described in paragraph 1.5 of the Supplementary Information. DNA samples were used for the quantification of bacterial 16S rRNA gene copy number and *R. solani* using real-time PCR (qPCR) and digital PCR (dPCR). To determine the exact number of 16S rDNA gene copies present in soil samples, a calibration curve was constructed using dPCR. For this purpose, total DNA extracted from the bacterial strain B04A42 was used as template in a series of 1:5 serial dilutions. The primers used were 16S_1055F (5′-ATG GCT GTC GTC AGC T-3′) and 16S_1392R (5′-ACG GGC GGT GTG TAC-3′), and the TaqMan probe was 1115P (5′-[FAM]C AAC GAG CGC AAC CC[TAM]-3′). The digital PCR reaction mixture had a final volume of 12 µl and consisted of: 1X QIAcuity Master Mixes, 900 nM of each primer, 200 nM of the TaqMan probe, and 2 µl of DNA from each dilution. The amplification protocol was as follows: 2 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 15 s at 50 °C, and 30 s at 72 °C. Once the calibration curve was established, it was possible to associate the absolute copy number obtained by dPCR with the Ct value from qPCR performed on soil samples with a simple calibration line ($R^2 = 0.998$), thereby converting the Ct values into 16S gene copy numbers. Real-time analyses were performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster, California, USA). Soil samples were diluted to 10 ng μl^{-1} of DNA and amplified in duplicate. The composition of the reaction mixture was identical to that used in dPCR, except for TaqMan™ Universal PCR Master Mix, no AmpErase™ UNG (Applied Biosystems), which was used at a final concentration of 1X. The thermal cycles conditions were the same as described above. Detection of *R. solani* in soil samples through qPCR was performed using 2 µl of DNA (1:50 dilution) in a reaction mix composed of: 1X of Power SYBR™ Green PCR Master Mix, 300 nM of each primer STRS1(F) (5′-AGT GTT ATG CTT GGT TCC ACT-3′) and ITS4(R) (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Gardes and Bruns 1993; Caterino et al. 2024). The amplification protocol consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and combined annealing, extension and fluorescence acquisition at 60 °C for 1 min. Each sample was amplified in duplicate and a subset of samples showing different Ct values after qPCR were quantified by dPCR, using a reaction mix containing 1X EvaGreen PCR Master Mix (FAM channel), 0.4 µM of each primer, and 2 µl of template DNA. The thermal cycle included an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 15 s. By comparing the outcomes of both assays, a calibration curve was established, linking the absolute copy number from the dPCR assay to the corresponding Ct value from the qPCR, thus deriving an equation to convert Ct into copy number: $y = -44.703x + 1374.3$. This calibration line was used for the quantification of *R. solani* in different treatment samples.

The same DNA was used for bacterial community analysis through long-read 16S sequencing of three replicates per treatment in the second and final *in vivo* trials. DNA libraries for Oxford Nanopore sequencing were produced starting from 15 µl of 27F/1492R amplicons. DNA was amplified with 16S universal primers 27F (5′-AGA GTT TGA TCA TGG CTC A-3′)/1492R (5′-TAC GGT TAC CTT GTT ACG ACT T-3′) as previously described (Heuer et al. 1997; Yu et al. 2005). PCR products were initially purified following the Agencourt AMPure XP PCR Purification protocol (Beckman Coulter, Inc). Sequencing was carried out according to the Native Barcoding Kit 24 V14 protocol (Oxford Nanopore Tecnologies) using a pool of 24 samples per library. The sequencing device used was a MinION Mk1C, and libraries were loaded on a R10.4.1 flow cell (FLO-MIN114). The run duration was set at 24 h, with a minimum read length filter of 1000 bp, as the expected fragment length is around 1400 bp. The standard minKNOW basecalling generated a fastQ file which was analyzed

using the "Fastq 16S" algorithm on EPI2ME software version 5.2.3. Taxonomy assignment parameters included a minimum coverage of 80% and a minimum identity of 88%. Only reads between 1000 and 2000 bp were retained for analysis. Output data were processed and formatted into an OTU table. Microbiota analyses in R version 4.3.3 (R Development Core Team 2013) began with the generated OTU table. The Phyloseq (version 1.46.0) (McMurdie and Holmes 2013), vegan (version 2.6.4) (Oksanen et al. 2015) and ggplot2 (version 3.5.1) (Wickham 2016) packages were used for data handling and visualization. The sequencing reads utilized in this analysis are available at NCBI at PRJNA1338487.

Statistical analysis

Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene's tests, respectively. Data on germination percentages, dry weights, root systems, mean leaf area and *R. solani* quantification were compared between treatments using one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$), performed with SPSS Statistics v29 (IBM, Armonk, NY, USA). For datasets that did not satisfy the prerequisites for a parametric test, Kruskal-Wallis non-parametric test was used instead, followed by Dunn's test for multiple comparisons. This analysis was applied to the quantification data of bacterial 16S rRNA gene copy number and Growth Inhibition Percentage (GIP %) from the antifungal assays. Correlation analyses were performed using Spearman's rank correlation-. In the analysis of the microbial communities, Beta diversity was assessed by PCoA based on Bray-Curtis dissimilarity matrices, and the statistical relevance of the treatment factor was evaluated using PERMANOVA through the Adonis function of the vegan package, based on 10,000 permutations.

Results

In vivo antifungal assay against *Rhizoctonia solani* under greenhouse conditions

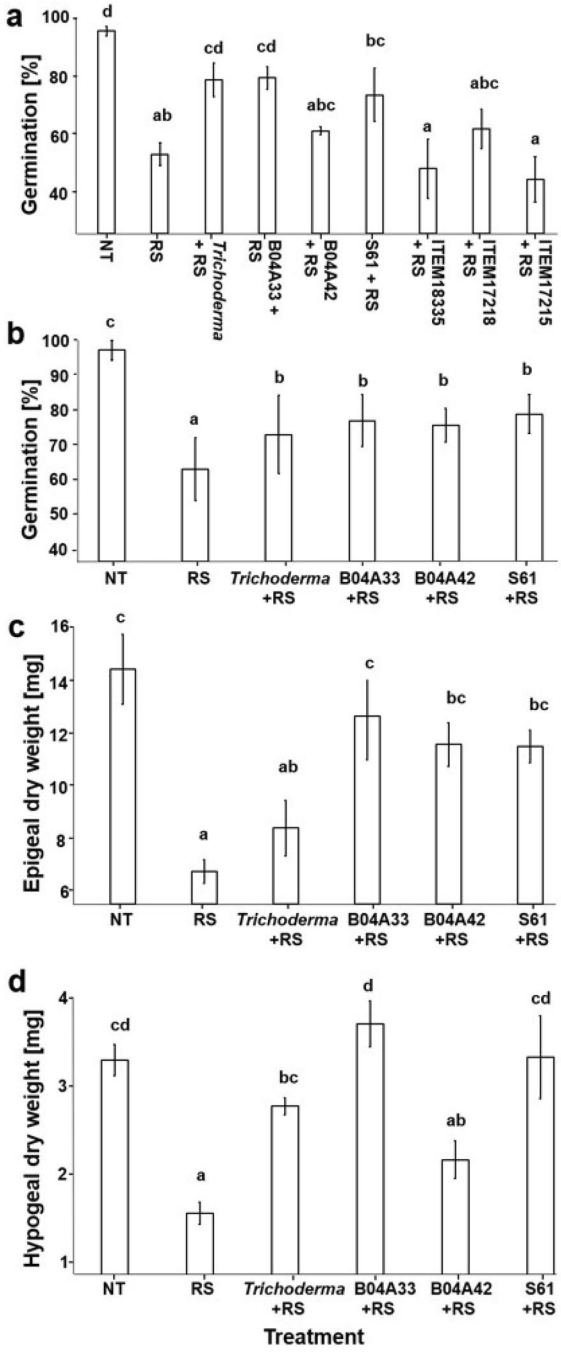
Results of the preliminary *in vitro* characterization (biochemical traits, antifungal activity, and effects on seed germination) are provided in Table 1. Additional results of these *in vitro* characterization assays are provided in Supplementary Information (Sects. 2.1–2.3, and Supplementary Figure S1).

In vivo, germination rate was determined by counting uprooted seedlings at the end of each trial (Fig. 1a). Based on the first assay, strains S61, B04A33, and B04A42 were selected for two additional trials. In Trials 2 and 3, germination trends were consistent with the initial results: B04A33 + RS and S61 + RS significantly increased germination compared to the positive control (RS). Data from these trials were pooled for statistical analysis. As shown in Fig. 1b, both treatments significantly improved lettuce germination in infested soil, with efficacy comparable to the commercial *Trichoderma*-based product, indicating antagonistic activity against *Rhizoctonia solani*.

Shoot dry weight (Fig. 1c) was lowest in the RS treatment, and similarly reduced under Trichoderma + RS. In contrast, bacterial treatments significantly enhanced aerial biomass, reaching values comparable to the non-treated control (NT). Root biomass (Fig. 1d) increased under all bacterial treatments except B04A42, suggesting improved seedling vigor and physiological performance following bacterial inoculation.

Image-based assessment of plant growth (and morphological responses) to bacterial treatments and *R. solani* inoculum

By analysing the images acquired six times per day, it was possible to monitor the growth of the epigeal part over time and observe the differences in germination patterns between the treatments. Figure 2a shows seed germination timing, while Fig. 2b shows cumulative leaf area expressed as the surface area (mm^2) of the aerial part over time. Seedlings grown in soil without *R. solani* inoculum germinated rapidly, with most seeds emerging within 80 h after sowing. In contrast, germination was delayed in all treatments where the pathogen was present in the soil. Most seedlings emerged within 100 h after sowing, but no significant differences in germination rates were observed between the RS control, the bacterial treatments and the *Trichoderma* treatment, except for S61 which showed higher germination than other infected treatments. Thus, while the bacterial strain did not accelerate germination, the



presence of *R. solani* delayed it. Seedlings grown in soil inoculated with bacterial strains had a comparable germination rate as *Trichoderma* treatment and showed a higher cumulative vigor (higher surface area covered [mm^2]) in leaves. These results suggest that the soil bacterial treatments enhanced the

Fig. 1 Effect of different bacterial treatments on lettuce seed germination and biomass production after growth in soil artificially inoculated with *R. solani* (RS). Bar plots show the mean value (± SD) of percentage of germinated seeds of four replicates for each treatment (**a**, **b**), above-ground dry weight (**c**) or below-ground dry weight (**d**). "NT" indicates the untreated control (no RS or bacterial inoculum), while "RS" is the positive control with *R. solani*. The other treatments combine *R. solani* with each bacterial strain or with a commercial *Trichoderma*-based biocontrol product in the first trial (**a**) and with selected bacterial strains in the second and third trial, combined in a single graph (**b**–**d**). Different letters above the bars indicate statistically significant differences among treatments (ANOVA followed by Tukey's post-hoc test, $p < 0.05$): **a** $F_{8,22} = 16.43$, $p < 0.001$; **b** $F_{5,44} = 19.29$, $p < 0.001$; **c** $F_{5,12} = 13.911$, $p < 0.001$; **d** $F_{5,12} = 20.3$, $p < 0.001$

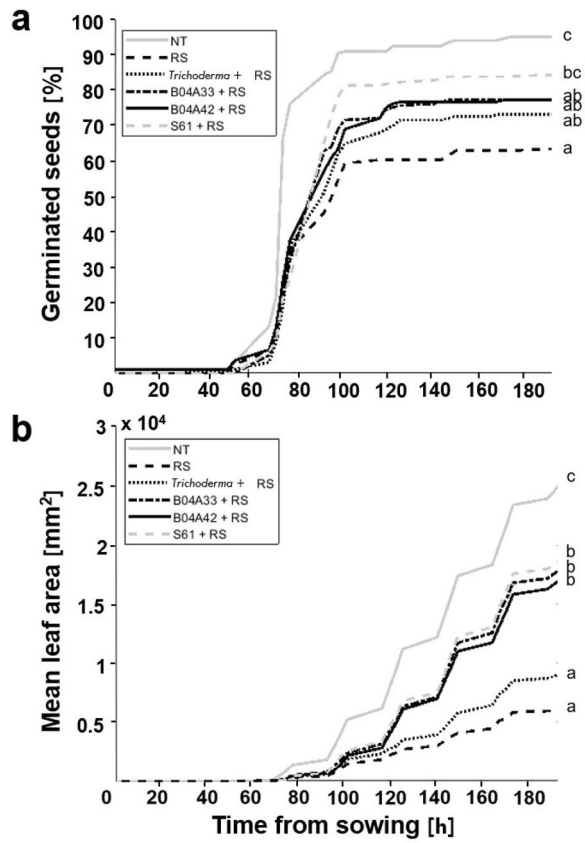


Fig. 2 Image-based analysis of seed germination dynamics, and seedling vigor over time in lettuce of four replicates for each treatment, during the last biocontrol trial. **a** Percentage of germinated seeds over time (hours from sowing). **b** Mean leaf area (mm^2) over time as a measure of cumulative seedling vigor. Different letters indicate statistically significant differences among treatments at the final time point (ANOVA followed by Tukey's post-hoc test, $p < 0.05$): **a** $F_{5,18} = 5.753$, $p = 0.002$; **b** $F_{5,18} = 15.276$, $p < 0.001$

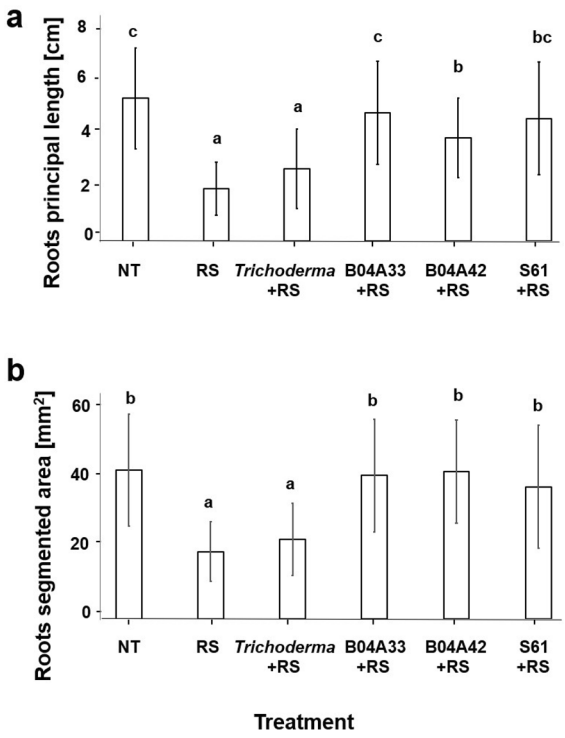


Fig. 3 Image-based analysis of root-related parameters of 49 seedlings for each treatment. Bar plots indicate the mean value (± SD). Treatments include: NT (non-treated control), RS (*Rhizoctonia solani*), Trichoderma + RS, B04A33 + RS, B04A42 + RS, and S61 + RS. Different letters above the bars indicate statistically significant differences among treatments (ANOVA followed by Tukey's post-hoc test, $p < 0.05$). **a** Measure of the principal root's length (mm). ANOVA: $F_{5, 234} = 21.98$, $p < 0.001$. **b** Area of the main structure of the segmented root system (segmented root area mm^2). ANOVA: $F_{5, 234} = 19.289$, $p < 0.001$

ability of the shoots to accumulate biomass during the early stages of development in the presence of pathogen. The presence of *R. solani* (RS) significantly decreased primary root length compared to the non-treated control (NT), while co-application with bacterial strains (B04A33, B04A42, S61) mitigated this effect resulting in lengths statistically similar to those of the untreated control, except for B04A42 treatment (Fig. 3a). The application of *Trichoderma* had no effect on root growth. The segmented root area data (Fig. 3b) mirrored the trend observed for root length. In this case RS and Trichoderma + RS resulted statistically different from untreated (NT) and bacterial strains (B04A33, B04A42, S61) treatments, confirming enhanced root system growth in the presence of bacterial strains. Data from the two root-related parameters confirm the trend of hypogeal dry weights: heavier weight corresponds to longer primary root and to and larger area of the main structure of the segmented root system (root segmented area). The Spearman rank correlation between manual and automated counting of emerged seedlings resulted in a Spearman's $R = 0.944$ ($p < 0.001$), indicating a good level of agreement and therefore a potential use of the method for seedling germination. Dry weights of the above part and leaf surface area follow approximately the same trend (Fig. 1c, 2b). Correlation analysis of these two parameters resulted in a Spearman's $R = 0.712$ ($p < 0.001$). The same procedure was applied to compare the hypogeal dry mass (mg) and the root segmented area (mm^2), resulted in a low correlation (Spearman's $R = 0.315$, $p < 0.001$).

Soil analysis

Quantitative real-time PCR (qPCR) analysis indicated a significantly higher abundance of bacterial populations in soil samples from the untreated control (NT) and S61 + RS treatments. In contrast, the RS, Trichoderma + RS, B04A33 + RS, and B04A42 + RS treatments exhibited lower bacterial abundance in the analyzed soil samples (Fig. 4a). The results regarding the abundance of *R. solani* (Fig. 4b) in the different treatments indicate a higher presence of the pathogen in the soil inoculated with *L. plantarum* strain (S61 + RS), compared to the other bacteria-treated soils. Nevertheless, this treatment also exhibited the highest number of 16S rDNA copies and recorded the highest seedling germination rate in the last two trials. The two treatments with *Bacillus sp.* and *Psychrobacillus sp.* (B04A33 + RS and B04A42 + RS) showed the lowest presence of the fungus, suggesting an antagonistic effect of these two strains that limited pathogen proliferation. In contrast, the abundance of *R. solani* in the soil treated with *Trichoderma* was comparable to that of the positive control (RS), suggesting a limited antagonistic effect of the BCA fungus. This finding is consistent with the previously discussed results on seedling vigor and overall plant health. Finally, *R. solani* was not detected in the untreated soil, confirming its absence prior to inoculation.

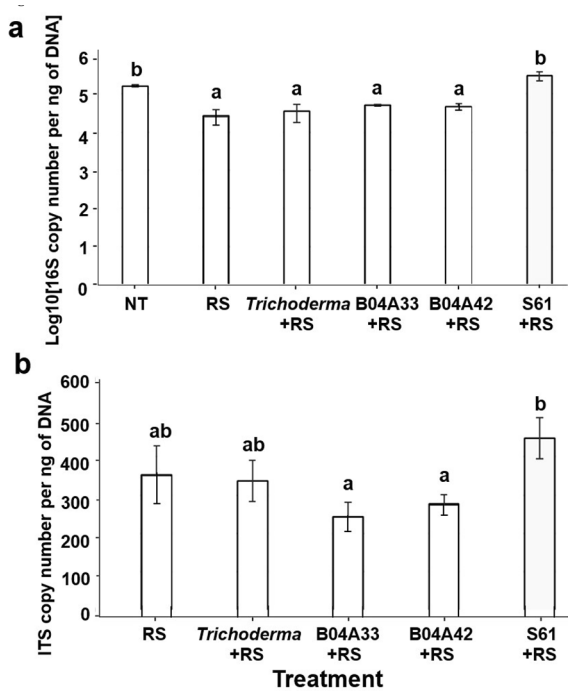


Fig. 4 **a** Number of 16S rDNA copies obtained from qPCR analysis on three replicates of DNA samples extracted from soil per treatment from the last two biocontrol trials. **b** Number of *R. solani* copies obtained from qPCR analysis on three replicates of DNA samples extracted from soil per treatment from the last two biocontrol trials. Treatments include RS (*Rhizoctonia solani*), Trichoderma + RS, B04A33 + RS, B04A42 + RS, and S61 + RS. Error bars represent SD. Different letters above the bars indicate statistically significant differences among treatments. For **a** Kruskal-Wallis *H* Statistic on 16S rDNA data: $\chi^2 = 19.326$, df = 5, $p = 0.002$, followed by Dunn's test for multiple comparisons; for panel **b** ANOVA: $F_{4,21} = 6.269$, $p = 0.002$, followed by Tukey's post-hoc test ($p < 0.05$)

Principal coordinate analysis (PCoA) of beta diversity based on Bray-Curtis dissimilarity followed by PERMANOVA did not reveal significant qualitative differences ($F_{3,15} = 0.938$, $p = 0.633$) among the OTUs identified by Nanopore sequencing across the various treatments (Fig. 5a). A predominant presence of bacteria belonging to the phylum *Pseudomonadota* was observed in all treatments, accounting for over 50% of the total bacterial reads (Fig. 5b). The relative abundance of other phyla varies only slightly between treatments. However, *Bacillota* was more prevalent in S61 + RS and B04A42 + RS treatments, with around 12.5% and 8.3%, respectively. This may be explained by the fact that the bacteria inoculated into the soil

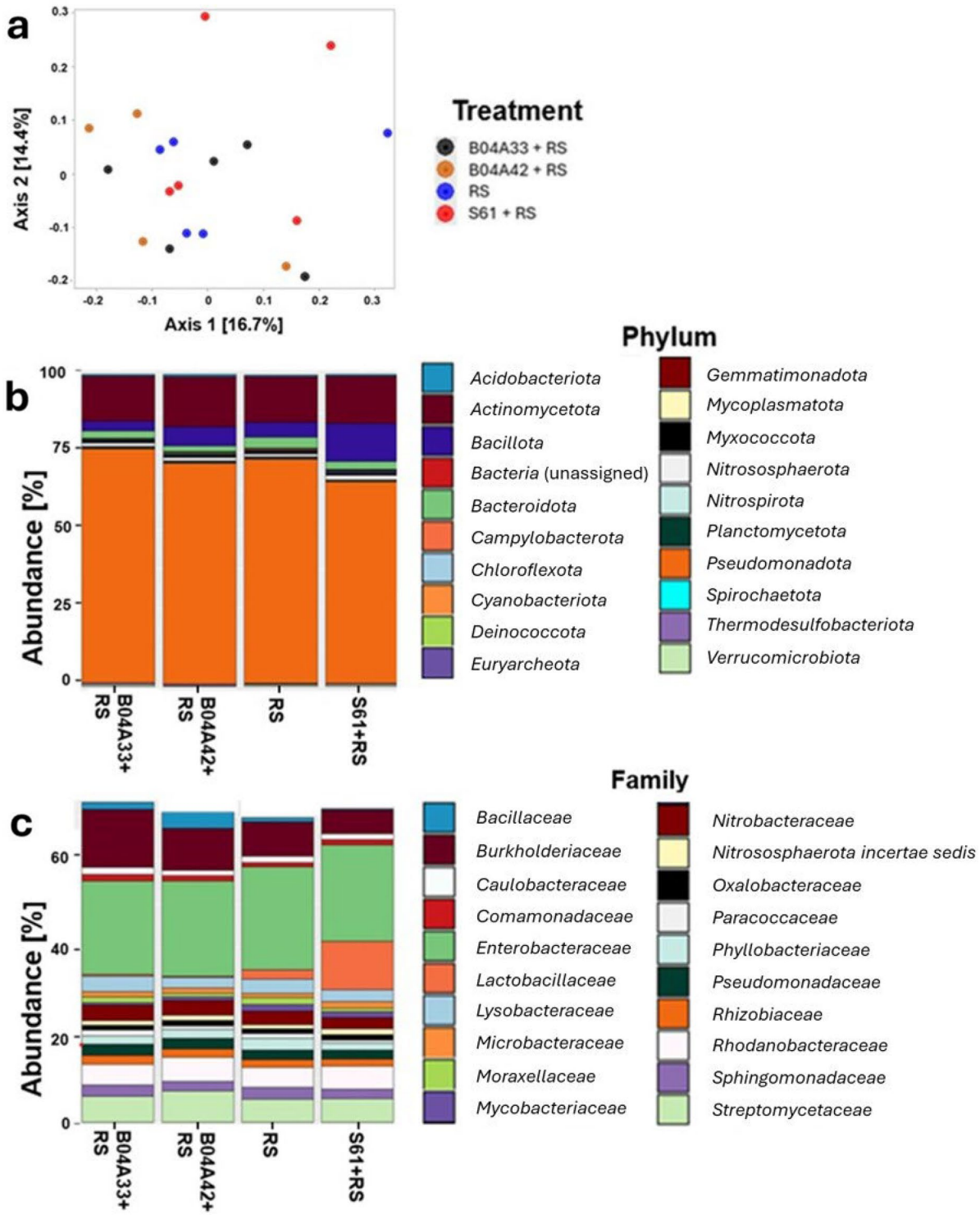
Fig. 5 **a** PCoA plot reporting the results of beta diversity, calculated with Bray-Curtis algorithm. Colour of the markers indicates the different treatments. Below graphs report the microbiota composition as relative abundance (%) at **b** phylum and **c** family levels. Both graphs show the treatments on the x-axis and the percentage of relative abundance on the y-axis. Bars reaching a total below 100% in **b** are due to OTUs assigned to families other than the most abundant 20 not being plotted on the graph. PERMANOVA (Bray-Curtis distance, 10,000 permutations, factor: treatment) performed on the PCoA showed no significant differences among treatments: $F_{3,15} = 0.938$, $p = 0.633$

belong to this phylum. Although the B04A33 strain also belongs to the phylum *Bacillota*, its abundance was lower than that observed in the pathogen only control (RS). At the family level (Fig. 5c), soils from the different treatments were primarily dominated by bacteria from the *Enterobacteriaceae* family. Notably, in soils treated with *Bacillus* spp. (B04A33 + RS) and *Psychrobacillus* spp. (B04A42 + RS), a higher relative abundance of Bacillaceae (~ 3% and ~ 5%, respectively) was observed compared to the positive control (~ 1%). Furthermore, in soil treated with the *L. plantarum* S61 strain, the Lactobacillaceae family ranked second in abundance (~ 10%), following *Enterobacteriaceae* (~ 20%).

Discussion

This study evaluated six bacterial strains for *in vitro* antagonism against four phytopathogenic fungi and for *in vivo* biocontrol of *Rhizoctonia solani*. A multidisciplinary approach integrating phenotypic assays, automated imaging, qPCR, and nanopore-based microbiota profiling was applied to assess efficacy and safety.

Among the strains, B04A33 showed the strongest in *vitro* activity, inhibiting *R. solani*, *Botrytis cinerea*, and *Fusarium verticillioides*. In greenhouse assays, it reduced disease incidence by 34% and increased seedling fresh weight by 216% compared to the positive control. These findings are consistent with previous reports on *Bacillus* spp., including *B. velezensis*, *B. subtilis*, and *B. amyloliquefaciens*, widely recognized for biocontrol efficacy (Abbas et al. 2017) and for which mechanisms of action are known. For example, *B. amyloliquefaciens* produces lipopeptides (surfactin, fengycin, bacillomycin D) that enhance plant defense (Chowdhury et al. 2015), while *B.*


a
Axis 2 [14.4%]
Axis 1 [16.7%]
Treatment
B04A33 + RS
B04A42 + RS
RS
S61 + RS
b
Abundance [%]
Phylum
Acidobacteriota
Actinomycetota
Bacillota
Bacteria (unassigned)
Bacteroidota
Campylobacterota
Chloroflexota
Cyanobacteriota
Deinococcota
Euryarcheota
Gemmatimonadota
Mycoplasmatota
Myxococcota
Nitrososphaerota
Nitrospirota
Planctomycetota
Pseudomonadota
Spirochaetota
Thermodesulfobacteriota
Verrucomicrobiota
RS
B04A33+ RS
B04A42+ RS
S61+RS
c
Abundance [%]
Family
Bacillaceae
Burkholderiaceae
Caulobacteraceae
Comamonadaceae
Enterobacteraceae
Lactobacillaceae
Lysobacteraceae
Microbacteraceae
Moraxellaceae
Mycobacteriaceae
Nitrobacteraceae
Nitrososphaerota incertae sedis
Oxalobacteraceae
Paracoccaceae
Phyllobacteriaceae
Pseudomonadaceae
Rhizobiaceae
Rhodanobacteraceae
Sphingomonadaceae
Streptomycetaceae

subtilis RB14 suppresses damping-off via iturin A and surfactin (Asaka and Shoda 1996).

The remaining strains (*Psychrobacillus* sp. and *Lactiplantibacillus plantarum*) exhibited typical plant growth–promoting traits (e.g., siderophore production, phosphate solubilization, H_2O_2 degradation) and strong antifungal activity against *F. verticillioides* (50–100%) and *B. cinerea*, with complete inhibition by *L. plantarum*, consistent with previous findings (De Simone et al. 2021). Antifungal effects of *L. plantarum* are attributed to organic acids and other antimicrobial metabolites (Suskovic et al., 2010; Chen et al. 2023), and the species is considered safe for agricultural use (Yilmaz et al. 2022). Instead, *Psychrobacillus* sp. have shown antifungal activity, though mechanisms remain unclear (Toral et al. 2021).

Only strain B04A33 inhibited *R. solani in vitro*. Despite lacking *in vitro* activity, both B04A42 and S61 reduced disease incidence and improved lettuce germination and vigour during *in vivo* experiments.

Germination under B04A33, B04A42, and S61 was comparable to or higher than the commercial *Trichoderma*-based treatment. Shoot and root biomass increased significantly under bacterial treatments relative to the infected control, except for root weight in B04A42 + RS. Notably, qPCR quantification did not show a significant reduction in *R. solani* soil abundance, suggesting that symptom mitigation may result from indirect mechanisms—enhanced plant defense, improved nutrition, or rhizosphere competition—rather than direct pathogen suppression.

Microbiota analysis revealed no clear beta diversity clustering, but treatment-specific shifts in taxonomic abundance were observed. B04A42 and S61 increased the relative abundance of Bacillota, particularly Bacillaceae and Lactobacillaceae, respectively, while B04A33 specifically enriched Bacillaceae. Monitoring persistence of inoculated taxa is essential, as survival of microbial inoculants is often limited by native community competition (Papin et al. 2024), although repeated application may enhance establishment (Mallon et al. 2018).

Automated RGB image analysis strongly correlated with manual germination counts (R = 0.944) and leaf area predicted shoot biomass (R = 0.712), supporting the reliability of image-based phenotyping for large-scale assays (Colmer et al. 2020; Iradukunda et al. 2024). The low-cost system effectively managed plant overlap and enabled dynamic assessment of germination and seedling vigour. Root imaging was less predictive of biomass, likely due to sample preparation constraints.

Overall, these results support further investigation of the selected strains to clarify their modes of action and assess their integration into sustainable crop management systems. The proposed automation workflow may be particularly valuable for greenhouse and vertical farming applications.

Author contributions Conceptualization: PC, RO; Formal analysis: AF, MT, PT, AP; Investigation: AF, MT, MG; Methodology: AF, PC, RO, SM; Visualization: AF, MC, AP; Writing – Original Draft: AF, MT, PC; Writing – Review and Editing: SM, MG, AP, AA, PT, MB, RO; Resources: MB, AA, PC, RO; Data Curation: AF, MT, AP; Funding Acquisition: RO, PC.

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Data availability The datasets generated for this study can be found in the manuscript itself and on NCBI's SRA at the BioProject number PRJNA1338487.

Declarations

Ethical approval The authors declare no competing interests. The study did not involve human or animal subjects.

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