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Research

Seed-Borne Endophytic Bacteria of Tomato: Dynamic Colonization and Implications in Plant Health

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Abstract

Seed-borne endophytic bacteria are essential for seed germination, plant growth, and stress tolerance. However, their functional diversity remains elusive because of difficulties in maintaining sterile conditions and amplifying the bacterial community without amplifying the plant genome. Using tomato (*Solanum lycopersicum* L.) as a model, we designed a novel culture system to study this bacterial community from seed to seedling, investigating its colonization and impact on plant health. Our workflow combined metabarcoding and culture-based approaches to capture the most accurate picture of the tomato seed-associated bacteria. An advanced metabarcoding protocol involving sequencing of the 16S-ITS-23S rRNA operon and the use of newly designed peptide nucleic acid clamps specific for tomato var. Moneymaker revealed a core microbiota consistently transmitted from seeds to shoots and roots. Key bacteria included *Ralstonia pickettii*, *Sphingomonas aerolata*, *Bradyrhizobium* spp., *Paucibacter aquatile*, *Microbacterium* spp., and *Stenotrophomonas* spp. Culture-dependent approaches isolated endophytic strains such as *Rummeliibacillus stabekisii*, *Peribacillus frigoritolerans*, and *Pseudomonas protegens*, which increase plant osmotic stress resistance. Using quantitative PCR, confocal microscopy, and colony counting, we demonstrated that *P. protegens* tagged with mCherry colonizes root, shoot, and leaf tissues. Overall, our study supports the hypothesis that a rich and stable core of endophytic bacteria, capable of enhancing drought resistance, is transferred from seeds to plant organs. Our study provides the most detailed investigation to date of the diversity of the seed-borne microbial community of tomato and offers new approaches to better assess the diversity of plant-associated microorganisms, a key step to improve crop resistance and productivity.

Keywords: 16S-ITS-23S *rrn* amplicons, community, core microbiota, endophytes, endophytome, metabarcoding, PNA clamps, tomato (*Solanum lycopersicum*) var. Moneymaker, vertically transmitted bacteria

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Plants are increasingly being recognized not as individual entities but as complex ecosystems known as "plant holobionts." These holobionts consist of the plant itself and its associated microbiota, which is highly diversified and inhabits various plant microhabitats, such as the rhizosphere, phyllosphere, and endosphere (Hassani et al. 2018; Lemanceau et al. 2017; Müller et al. 2016). The understanding of endophytes, a crucial component of these holobionts, has significantly evolved. Initially seen as harmless plant inhabitants, endophytes are now recognized as a diverse community of microorganisms that inhabit plants either obligatorily or facultatively, interacting dynamically within their hosts (De Bary et al. 1866; Lengrand et al. 2024). Endophytes, which encompass bacteria, fungi, archaea, and viruses, form a complex network of interactions that contribute to plant adaptation and survival in diverse environments. Indeed, several microbial endophytes have biostimulant properties (Pati and Rathore 2024). It is important to note, however, that not all endophytes are beneficial to the plant; some may have neutral or even detrimental effects on their host (Hardoim et al. 2015). Endophytes can be transmitted vertically (i.e., from parent to offspring via seeds) or horizontally (i.e., acquired from the environment), highlighting the flexibility of plant–microbe interactions (Hardoim et al. 2015; Truyens et al. 2014). Although extensive research has illuminated the microbial dynamics within shoot and root habitats, the seed endophyte microbiome remains less explored (Nelson 2018; Sánchez-López et al. 2018; Truyens et al. 2014).

Studying seed microbiota is crucial, as it constitutes the primary microbial inoculum for plants, offering early advantages that enhance growth and resilience to stresses, impacting subsequent plant generations (Rosenblueth et al. 2012; Truyens et al. 2014; Verma and White 2019). Some taxa, such as *Pantoea agglomerans*, *Pseudomonas viridiflava*, and *P. fluorescens*, are commonly found in the seeds of many plant species (Simonin et al. 2022). However, research on seed microbiota faces challenges, such as differentiating true endophytes from contaminants and understanding the dynamics and specific roles of microbial communities. Additionally, inconsistencies in methodology for sample preparation and sequencing make it challenging to compare findings across studies.

Tomato (*Solanum lycopersicum*) is one of the most significant agricultural crops, with global production reaching 186.1 million metric tons in 2022 (FAOSTAT 2024). Although it plays a crucial biological role in the plant, the seed microbiota of tomatoes remains largely unknown. Bergna et al. (2018) used 16S V4 sequencing to compare seed and root endophytic communities, but most studies focus solely on seed microbiota (Acuña et al. 2023; Flemer et al. 2022; Simonin et al. 2022) or rely on cultivation-based methods (López et al. 2018; Shaik and Thomas 2019; Sharma et al. 2021; Thomas and Shaik 2020; Tian et al. 2017; Xu et al. 2014). Typical cultivable members of seed-transmitted endophytic bacteria include *Bacillus*, *Micrococcus*, *Paenibacillus*, *Pantoea*, and *Pseudomonas* (López et al. 2018; Sharma et al. 2021). A thorough understanding of the tomato seed microbiota and its functions is essential to unlocking its potential impact on plant health and productivity. Indeed, despite the crucial biological role of the tomato seed microbiota, such as the potential to improve growth of the host (Tian et al. 2017; Xu et al. 2014), improve host plant metal tolerance, suppress plant pathogens (López et al. 2018; Marzouk et al. 2021; Morella et al. 2019; Sharma et al. 2021), or facilitate uptake of nutrients (Bergna et al. 2018), many questions remain unanswered in this field. For instance, what is the composition of the core microbiota that is transmitted from the seed to all plant organs? It is also unclear whether all seed-borne endophytic bacteria can colonize various microhabitats, such as roots and shoots, or if there is a distinct distribution within each microhabitat. Another important question is whether seed-borne endophytic bacteria transition to becoming epiphytes, and if the same genera are involved in epiphytic colonization in both shoots and roots. Furthermore, it remains to be determined whether certain isolated endophytic bacteria are able to recolonize the plant when coated on seeds or applied to roots, the pathways they use for colonization, and whether they can colonize all plant organs or are confined to specific microhabitats.

In this study, we set out to establish the most comprehensive microbiota profile of tomato seed-transmitted endophytic bacteria in seed and growing plants (Fig. 1). We first developed a sterile culture system approach that enabled us to accurately characterize the seed-transmitted bacterial microbiota of *S. lycopersicum* var. Moneymaker (Section "Plant material and sterile growth conditions"). During seed germination, seed-borne bacteria may either colonize the plant as endophytes or exit to become epiphytes or rhizosphere colonizers (Hardoim et al. 2012). To explore these dynamics, we used newly designed peptide nucleic acid (PNA) clamps and 16S-ITS-23S rRNA operon (*rrn*) sequencing to analyze bacterial communities in both seeds and plants, which were either subjected to surface disinfection or left untreated (Section "Identification of seed-transmitted endophytic bacteria by metabarcoding"). Indeed, one of the primary challenges in studying endophytic bacterial communities is amplifying bacterial DNA, which is often masked by the abundance of plant DNA (Arenz et al. 2015; Lundberg et al. 2013). Although universal PNA clamps are useful, they do not entirely block plant DNA, particularly in cases in which plastid DNA integrates into the nuclear genome, with mutations altering the target sequences (Sheppard and Timmis 2009; Zhang et al. 2020). Additionally, second-generation sequencing technologies such as Roche 454 GS FLX and Illumina MiSeq provide short reads, limiting taxonomic resolution and making it difficult to distinguish closely related species (Goodwin et al. 2016). By contrast, third-generation sequencing platforms such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies used to sequence full 16S-ITS-23S *rrn* amplicons offer long reads that enhance taxonomic resolution, and allow for a detailed analysis of microbial communities at the species or strain level (Cuscó et al. 2019).

We also employed culture-dependent methods to investigate seed-transmitted endophytic bacteria in various seed and plant organs (Section "Identification and characterization of seed-transmitted endophytic bacteria by culture-dependent method"). Based on this methodology, we identified and assessed the plant growth-promoting (PGP) traits of the isolates in vitro. Additionally, we evaluated the effects of selected isolates on plant vigor in planta (Section "Characterization of cultivable bacterial endophytes"). Finally, we examined the colonization patterns of two previously isolated endophytic bacteria by coating seeds with mCherry mutants, tracking them in plants using quantitative polymerase chain reaction (qPCR), colony counting, and confocal analysis (Section "Plant colonization assays with TN7 mutant strains").

Materials and Methods

Plant material and sterile growth conditions

To obtain the most accurate profile of seed microbiota composition and avoid contamination by environmental microbes, we first established a protocol for tomato cultivation in sterile conditions. Tomato seeds (*S. lycopersicum* var. Moneymaker) were surface-sterilized by immersing in <5% sodium hypochlorite for 10 min under orbital shaking and rinsed three times with sterile distilled water for 20 min. Sterility of surface disinfection was checked by plating disinfected seeds on Luria broth agar (LBA)

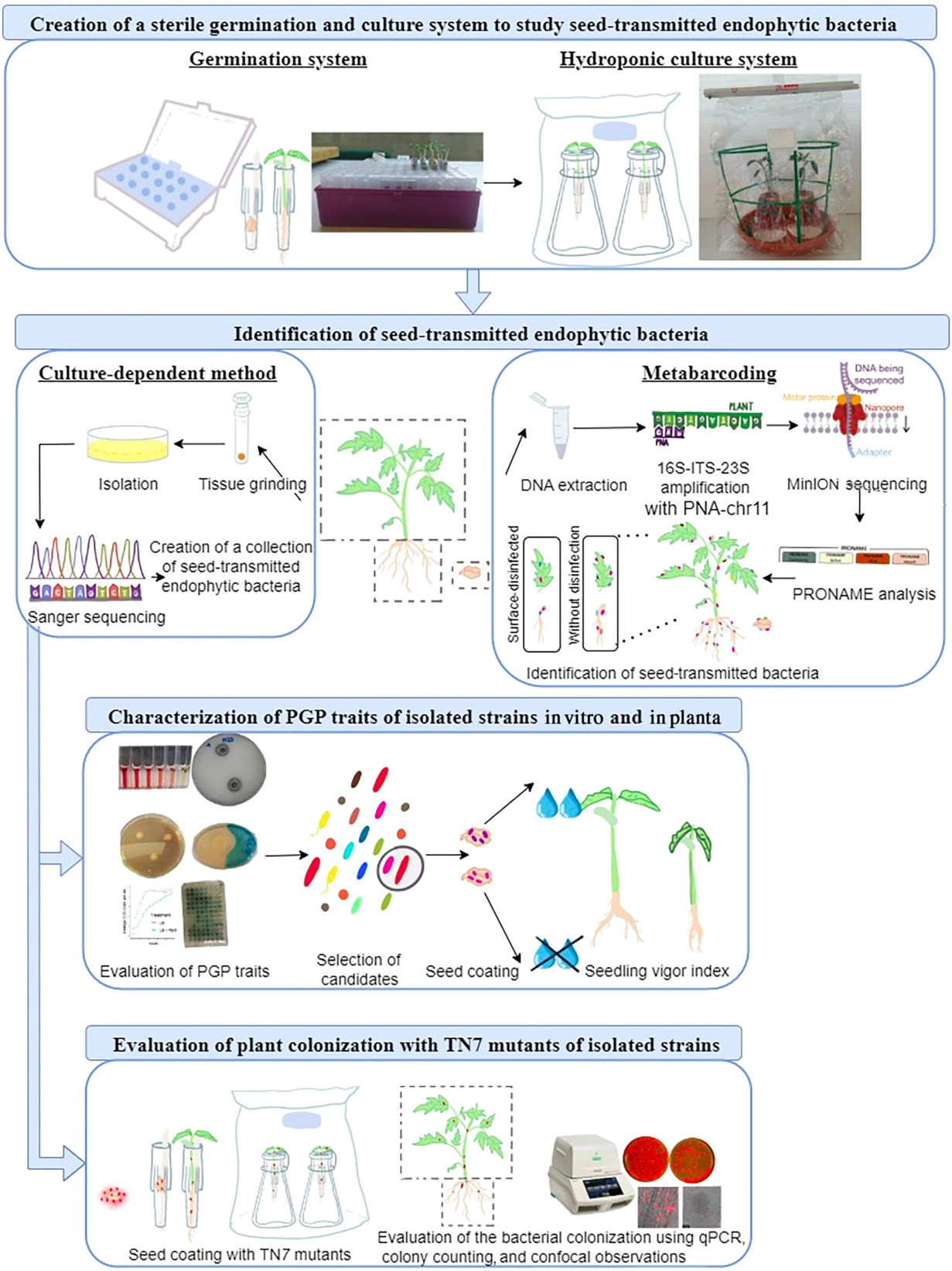

FIGURE 1

Schematic of the complete experimental workflow. A sterile germination and culture system was developed to investigate seed-borne endophytic bacterial communities in seeds, roots, and shoots using both metabarcoding and culture-dependent methods. The resulting collection of seed-borne endophytic bacteria enabled the study of their plant growth-promoting (PGP) traits *in vitro* and *in planta* and allowed the creation of TN7 mutants for tracking bacterial colonization in plants through quantitative PCR (qPCR), colony counting, and confocal microscopy.

plates, which were monitored for 2 weeks to check for bacterial growth (Fitzpatrick et al. 2018a). Three aliquots of approximately 150 to 200 seeds were reserved at −80°C for the analysis of endophytic bacterial communities. The remaining seeds were germinated in a sterile box for 10 days (7 days in the dark and 3 days under light, photoperiod 16/8 h) in a germination chamber at 22°C and 70% relative humidity. At the two-cotyledon stage, the seedlings were transferred into a custom-designed sterile hydroponic system, which consisted of two Erlenmeyer flasks (150 ml) filled with Hoagland's solution adapted for tomato plants (NH_4NO_3: 0.04 g liter^{-1}; $Ca(NO_3)\cdot 4H_2O$: 0.413 g liter^{-1}; KNO_3: 0.2035 g liter^{-1}; KH_2PO_4: 0.137 g liter^{-1}; $MgSO_4\cdot 7H_2O$: 0.123 g liter^{-1}; $MnSO_4\cdot 5H_2O$: 0.265 mg liter^{-1}; H_3BO_3: 0.7 mg liter^{-1}; $CuSO_4\cdot 5H_2O$: 0.075 mg liter^{-1}; $(NH4)Mo_7O_{24}\cdot 4H_2O$: 0.004 mg liter^{-1}; $ZnSO_4\cdot 7H_2O$: 0.3 mg liter^{-1}; Fe EDDHA: 0.03 g liter^{-1}). This system was enclosed in an autoclavable culture bag with a 0.02-µm filter (Sun bag, transparent, B7026-100EA; Sigma-Aldrich) to allow gas exchange supported by a plastic frame and a sterile plant potholder, ensuring a controlled environment (Fig. 1; Supplementary Fig. S4). Hydroponic solution was open to the plant shoot. Tomato plants were cultivated in a growth chamber under a 16/8-h (light/dark) photoperiod regimen, 70% relative humidity, and a temperature of 24/22°C (day/night). A detailed description and images of the germination and culture systems are provided in Supplementary Figures S3 and S4. The hydroponic solution was replenished under sterile conditions every week using a horizontal laminar flow hood to prevent contamination. During the process, only the exterior of the culture bags was handled, and the solution was aspirated using a sterile pipette gun and 25-ml sterile tips, which were replaced between each plant to maintain sterility. The Erlenmeyer flasks were then refilled with freshly autoclaved hydroponic solution, and each culture system was treated individually to avoid cross-contamination between treatments. Control systems without plants were also refreshed at the same frequency to ensure consistency and validate the sterility of the system. After 3 weeks of growth in the hydroponic system, the plants were harvested for DNA extraction and bacterial isolation. By this time, they had reached the four-leaf stage. Our objective was to grow well-developed plants rather than seedlings while preserving sterile conditions. Three weeks was the longest period during which the plants remained in good condition in the sterile culture system used.

The sterility of the system was checked at the harvest time by spreading hydroponic solution of a system without plant on LBA medium. Moreover, the possible contamination of the hydroponic solution with noncultivable bacteria was evaluated using a molecular method: the DNA of hydroponic solution was extracted using a hexadecyltrimethylammonium bromide (CTAB) chloroform protocol followed by PCR targeting the 16S rRNA gene.

Identification of seed-transmitted endophytic bacteria by metabarcoding

DNA extraction from seeds, shoots, and roots. To identify seed-transmitted endophytic bacteria, map their distribution within plant organs, and determine their colonization mode (i.e., endophytic versus epiphytic), we analyzed the bacterial communities of seeds, roots, and shoots (encompassing all aerial parts) from both surface-disinfected and nondisinfected plants. Three aliquots of disinfected seeds (approximately 150 to 200 seeds per aliquot) were immersed separately in liquid nitrogen and ground under sterile conditions. Meanwhile, three plants were directly separated into shoots and roots and ground in liquid nitrogen and three other plants underwent surface disinfection before processing to identify seed-borne endophytes and epiphytes. Disinfection was performed on the entire plant to prevent the disinfectant solution from penetrating the interior. Tomato plants were first placed in nucleic acid preservation buffer (124 mM Na_2HPO_4) and sonicated in an ultrasonic cleaning bath (VWR) for 1 min to dislodge surface bacteria. Subsequently, plants were submerged in a sodium hypochlorite solution (5.25%) for 6 min and rinsed with sterile water (Ruiz-Pérez and Zambrano 2017). The intact plants were then dried on sterile paper under sterile conditions. To ensure thorough sterilization, we imprinted the plants on LBA plates, which were monitored for 2 weeks to check for bacterial growth (Fitzpatrick et al. 2018a).

Following this, shoots and roots were separated and ground in liquid nitrogen. Each powdered sample, comprising three pools of seeds (approximately 150 to 200 seeds per aliquot), three surface-disinfected roots and shoots, and three nondisinfected roots and aerial parts, underwent DNA extraction using a modified CTAB-based method to facilitate plant tissue extraction. Briefly, NaCl (100 µl, 5 M) was added before isoamyl alcohol/chloroform to reduce high polysaccharide concentration in the extractant (Fang et al. 1992). The DNA pellet was diluted in 20 µl of sterile nuclease-free water.

PCR amplification of ribosomal markers of endophytic bacteria with universal PNA clamps and the new design, PNA_chr11. Extracted DNA was amplified using the primers 16S-8F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 23S-2490R (5′-CGACATCGAGGTGCCAAAC-3′) targeting the 16S-ITS-23S *rrn* (approximately 4,500 bp) (Karst et al. 2021). Their nonselectivity was tested by comparing the reads amplified with three other pairs of primers commonly used (Supplementary Table S6). PCR amplification included two universal PNA clamps (mPNA and pPNA) and a newly designed PNA_chr11 (CTGCTAATACC**Y**CG**K**AGGCTGA) clamp that suppressed almost completely tomato genome amplification with a proportion of reads attributed to plant of 0.14% compared with 60.2% without the new clamp. Details regarding the development of the new PNA can be found in Supplementary Information S1. The mPNA and pPNA clamps specifically bound to mitochondrial and chloroplast DNA, respectively, and inhibited amplification, whereas PNA_chr11 specifically detected a mutation in the tomato genome (var. Moneymaker) caused by plastid DNA integration into the chromosomal DNA. Using all three clamps is essential to minimize the amplification of tomato DNA. The PCR mix (25-µl total volume) contained 2× GoTaq Long PCR Master Mix (Promega), 0.4 µM of 16S-8F primer, 0.4 µM of 23S-2490R primer, 2 µM of mPNA (to block mitochondrial amplification), 2 µM of pPNA (to block plastid amplification), 2 µM of PNA_chr11 (to block chromosome 11 of *S. lycopersicum* genome amplification), and 20 ng of DNA (Lundberg et al. 2013). The PCR thermal profile consisted of an initial denaturation of 2 min at 95°C followed by 30 cycles of 20 s at 95°C, 60 s at 74°C, 30 s at 55°C, 5 min at 72°C, and a final extension of 10 min at 72°C. For each sample, PCR was performed in three replicates and pooled before purification to ensure homogeneity. The amplicons of the 16S-ITS-23S *rrn* were cleaned up with AMPure XP beads (Beckman Coulter) using a 0.5× bead-to-sample ratio. The amplicon concentration and quality were evaluated using a Quantus Fluorometer (Promega) and a NanoDrop spectrophotometer (Thermo Fisher Scientific), respectively. PCR products were visualized on a 0.8% agarose gel.

Library preparation and Oxford Nanopore sequencing. Libraries were prepared using Ligation Sequencing Kit V14 (SQK-LSK114; Oxford Nanopore Technologies) according to the manufacturer's instructions. Briefly, amplicons were processed for end repair using the NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs), and sequencing adapters

were attached. Libraries were sequenced for 24 h using Flongle Flow Cells (R10.4.1) and a MinION device (Oxford Nanopore Technologies).

Data processing and analysis of diversity. The raw data were processed using PRONAME (Dubois et al. 2024). Initially, the data were imported into PRONAME, where adapters and primers were trimmed. Subsequently, a graph illustrating the length versus quality distribution of simplex and duplex reads was generated. The data were then filtered to retain only duplex reads that were between 3,500 and 5,000 bp in length and had a minimum quality score of 15. PRONAME further clustered reads together by setting a 90% identity threshold and polished the centroid sequences with 'medaka' using 300 reads subsampled from each cluster. Finally, chimeric sequences were filtered out and the representative sequences were subjected to a BLAST analysis against the rEGEN-B database, a curated and extensive reference database dedicated to bacterial *rrn* sequences, also included in the PRONAME package.

The analysis of data composition was carried out using the 'phyloseq' package (McMurdie and Holmes 2013) in RStudio and the results were visualized using the 'ggplot2' package (Wickham 2011). For alpha diversity, the 'vegan' package was utilized to calculate the observed features as well as Shannon, Simpson, and inverse Simpson indexes, and the results were statistically evaluated using the Wilcoxon test with Benjamini-Hochberg adjustment for multiple comparisons.

Beta diversity was examined using principal coordinate analysis with the 'ape' package (Paradis and Schliep 2019) and further analyzed through the analysis of similarity test employing the 'vegan' package (Oksanen et al. 2022). Additionally, Venn diagrams were generated using the 'VennDiagram' package (Chen and Boutros 2011).

Identification and characterization of seed-transmitted endophytic bacteria by a culture-dependent method

Three pools of four seeds were weighed, cut with a sterile blade. The tomato plants were surface disinfected (see section "DNA extraction from seeds, shoots, and roots") prior to isolation of the endophytic bacteria. Six plants per treatment were separated into shoots (comprising all aerial parts) and roots after the surface disinfection. Seeds, shoots, and roots were placed individually in 2-ml tubes with phosphate-buffered saline (PBS) solution (NaCl: 8 g liter^{-1}; KCl: 0.2 g liter^{-1}; $Na_2HPO_4{\cdot}(12H_2O)$: 2.9 g liter^{-1}; KH_2PO_4: 0.2 g liter^{-1}) (European and Mediterranean Plant Protection Organization 2018). The tubes were homogenized twice at room temperature in a FastPrep-24 classic bead beating grinder and lysis system (MP Biomedicals) for 30 s and dilutions (10^{-1}, 10^{-2}, 10^{-3}) were spread on four different nutrient-rich levels of medium (nutrient agar, LBA, V8, and buffered charcoal yeast extract) (Wells et al. 1981) supplemented with an antifungal (cycloheximide 50 mg liter^{-1}) and incubated at 28°C for 2 weeks. Given that the tomatoes were grown in a sterile system, any bacteria present in the hydroponic solution must have originated from the seeds. Therefore, the hydroponic solution was sterilely pipetted at harvest and 100 µl was spread on the same medium (nutrient agar, LBA, V8, and buffered charcoal yeast extract).

For molecular identification, single colonies of each isolate were mixed in water and heated for 5 min at 100°C before ice shocking for bacterial DNA extraction. The 16S rDNA gene was PCR-amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR conditions were as follows: an initial denaturation for 5 min at 94°C, 30 cycles (1 min at 94°C, 30 s at 61°C, and 1 min 30 s at 72°C), 10 min at 72°C, and hold at 10°C. PCR products were checked on a 1.2% agarose gel and purified samples were sent to Microsynth for Sanger sequencing. All of the sequences obtained were analyzed for homology searches using the BLAST algorithm against the nucleotide sequences present in the National Center for Biotechnology Information (NCBI) GenBank database.

Random amplified polymorphic DNA (RAPD) of culturable bacteria was performed to verify the clonality of bacterial isolates using RAPD272 (5′-AGCGGGCCAA-3′) (Hematzadeh and Haghkhah 2021). The PCR conditions were as follows: an initial denaturation for 2 min at 94°C, 35 cycles (30 s at 94°C, 30 s at 35°C, and 2 min at 72°C), and 10 min at 72°C (Rosić et al. 2023). The profiles of strains with the same identification on NCBI BLAST were compared and the strains with the same identification, RAPD profile, and phenotype of colonies (size, shape, relief, contour, surface, and color) were considered clones.

A phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei 1987) in Geneious (2023.0). Precultures of the isolated bacteria were mixed with glycerol and stored at −80°C to form a collection reflecting the cultivable component of the tomato seed microbiota.

Characterization of cultivable bacterial endophytes

The initial screening of isolated bacteria for drought stress was conducted using the method described by Kumari et al. (2016). Briefly, bacterial precultures were incubated overnight at 28°C with shaking at 180 rpm. Subsequently, a 0.5-ml aliquot of each preculture was added to 4.5 ml of Luria-Bertani (LB) broth with or without 10% wt/vol polyethylene glycol (PEG) and incubated under shaking at 28°C for 5 days. Bacterial growth viability was monitored by measuring the optical density at 600 nm (OD 600). Strains that exhibited a growth ratio in LB versus LB + PEG equal to or greater than 1 and those between 1 and 0.5 were selected for further analysis.

For these selected isolates, a more detailed assessment of drought stress tolerance was performed by comparing their growth curves in LB and LB supplemented with PEG. Cultures were initially carried out with LB and then centrifuged to discard the supernatant. The bacterial cell pellets were resuspended in fresh LB or LB + PEG to an OD 600 of 0.01. Growth was monitored at 28°C for 24 h by measuring OD 600 hourly, with controls set up using noninoculated LB and LB + PEG. This test was conducted twice, in triplicate, using 96-well microplates (200 µl per well) on a Multiskan FC (Thermo Fisher Scientific). Growth inhibition under osmotic stress was quantified based on the area under the growth curves, with inhibition rates categorized as follows: less than 25% inhibition indicated high tolerance, 25 to 50% indicated moderate tolerance, and greater than 50% indicated low tolerance.

Bacterial isolates were tested for indole-3-acetic acid (IAA) production using a colorimetric technique performed with van Urk-Salkowski reagent according to the Salkowski method (Ehmann 1977) by adjusting OD 600 of the bacterial suspension at 0.1. A calibration curve was created with pure IAA (ranging between 0 and 100 µg ml^{-1}) diluted in LB (Gang et al. 2019). Uninoculated LB served as a control. The experiment was repeated three times.

Exopolysaccharide (EPS) production was estimated as described by Paulo et al. (2012) using solid medium-containing discs of filter paper (5-mm diameter) that were saturated with active cultures (5 µl of culture adjusted to OD 600 of 0.1). EPS production was evidenced after 7 days by the presence of mucoid colonies on the discs, which was confirmed by the formation of a precipitate when a part of the colony was mixed with absolute ethanol.

The ability of bacteria to solubilize precipitated phosphate was tested by inoculating 10 µl of an overnight bacterial culture in an artificial well on Pikovskaya medium containing calcium phosphate. The formation of a clear halo around the well after incubation at 30°C for 7 days indicated a positive result (Thomloudi et al. 2021).

Siderophore production was evaluated by the Chrome Azurol S (CAS) agar assay (Schwyn and Neilands 1987). A 10 µl volume of overnight culture was inoculated in the artificial well on a CAS agar plate (Louden et al. 2011). Released siderophores were detected by the formation of orange halos on CAS agar plates after incubation for 7 days at 30°C.

Assessment of bacterial coating effects on seed vigor index. The identification of selected strains for the in planta test was refined by performing a multilocus sequence analysis targeting, in addition to 16S rRNA, the gene *gyrB* for bacterial strains belonging to *Firmicutes* and the genes *rpoB*, *rpoD*, and *gyrB* for *Pseudomonas* strains. PCR amplification conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s, elongation at 72°C for 60 s, and a final extension step at 72°C for 5 min. The characteristics of primers and their annealing temperatures are detailed in Supplementary Table S7. Sequences were concatenated and queried against the NCBI RefSeq representative genomes database.

For the nine selected bacterial strains based on PGP tests, a single colony was grown overnight in LB (28°C, 180 rpm). Then, the bacterial cultures were centrifuged (5,000 rpm, 10 min), the pellet was washed three times with PBS, and the OD 600 was adjusted to a concentration of 10^8 CFU ml^{-1}. Tomato seeds were disinfected according to previously described methods before incubation in the bacterial suspensions for 2 h. Control seeds were similarly incubated in PBS alone. After coating, the seeds were placed on Whatman paper in Petri dishes, which were moistened with either sterile water or a 10% wt/vol PEG 6000 solution to induce osmotic stress of −0.2 MPa. Each treatment, whether nonstress or osmotic stress conditions, was performed in triplicate with 12 seeds per Petri dish. The dishes were incubated in the dark at 22°C for 7 days and then exposed to light for 3 days. After 10 days, the seedling vigor index was calculated using the formula described by Bhatt et al. (2015):

$$\text{Seed vigor index} = \%\text{germination} \times (\text{seedling root length} + \text{shoot length})\ (\text{cm})$$

Plant colonization assays with TN7 mutant strains

The colonization of two bacterial strains, *P. protegens* C1M8 and C1M39, was evaluated in tomato by genetically labeling these strains with mCherry. Transformation to express the mCherry fluorescent protein was performed following the recommendations of Ferreira et al. (2008) (Supplementary Information S3). Fluorescence of bacteria was evaluated by confocal analysis and plasmid insertion was verified by DNA extraction from bacterial strains via thermal shock (5 min at 100°C, 10 min on ice) and amplification of the *mCherry* gene with primers TN7_2377F (5′-ACCGTAACACGCCACATCTT-3′) and TN7_3033R (5′-GCAATTGCCTGGTGCATACAA-3′), applying the following thermal protocol: 5 min at 95°C followed by 30 cycles of 45 s at 95°C, 30 s at 62°C, and 45 s at 72°C and a final extension for 10 min at 72°C. TN*7* insertions take place at specific attachment sites located downstream of the coding region of the *glmS* gene and have been demonstrated to incur no fitness penalty on the bacterial host (Lambertsen et al. 2004).

To evaluate the colonization capacity of two bacteria in tomato plants, seed coating was performed as described earlier with the modification that the bacterial strains were grown in LB medium supplemented with spectinomycin. Ten days after germination, the assessment of bacterial endophytic and epiphytic colonization within various plant organs was conducted by qPCR, colony-counting assays, and confocal microscopy analysis. Three plants per bacterial strain were cut into leaves, shoots, and roots for direct analysis. Additionally, another set of three plants per treatment was surface-disinfected by submerging in a 5.25% sodium hypochlorite solution for 6 min followed by three rinses in sterile water.

Development of a qPCR assay to quantify TN7 mutants in tomato. To quantify TN*7*::mutant strains within various organs of *S. lycopersicum*, qPCRs were performed on plant DNA extracted using the CTAB method. Specific primers (F_TN7_25511 and R_TN7_2576) were designed to amplify TN*7* plasmid and confirmed for specificity through in silico analysis on Primer-BLAST (NCBI) and PCR amplification of inoculated samples. Because the TN*7* plasmid integrates at one position in the bacterial genome, it serves as a marker for bacterial colonization. Simultaneously, DNA quantification of *S. lycopersicum* was performed with primers TUB-F and TUB-R, targeting the β-*tubulin* reference gene (Løvdal and Lillo 2009). The sequences of primers and the thermal cycling conditions are detailed in Supplementary Table S8.

Standard curves were constructed by purifying PCR products for TN*7* and β-*tubulin* amplicons using the SmartPure PCR kit (Eurogentec). DNA concentrations were measured using the Qubit HS assay kit (Thermo Fisher Scientific), adjusted to 10^{10} copies, and serially diluted to create calibration curves correlating known DNA quantities with cycle threshold values.

The qPCR mixes contained 2× Takyon SYBR MasterMix dTTP (Eurogentec), 500 nM of each primer, and 2 µl of DNA template in a 20-µl reaction volume. Amplifications were performed on a CFX96 thermal cycler (Bio-Rad) in duplicate. Bacteria were quantified under the following conditions: initial denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 54°C for 15 s, and 72°C for 15 s. For TUB amplification, the annealing temperature was modified to 60°C.

The β-*tubulin* gene was confirmed to be a single-copy gene in the genome of *S. lycopersicum*. The number of β-tubulin copies was approximately 975 per nanogram of DNA, as the genome size of *S. lycopersicum* is approximately 950,000,000 bp. This allowed calculation of bacterial quantity per gram of plant DNA using the following equation:

$$\text{Bacteria (ng of plant DNA)}^{-1} = \frac{\text{Number of TN7 copies}}{\left(\frac{\text{Number of TUB copies}}{975}\right)}$$

For qPCR, three plants were surface-disinfected and three others were not in order to quantify epiphytic and endophytic bacterial colonization.

Colony-counting assay. A colony-counting assay was performed to confirm the presence of viable inoculated bacteria in the different organs of the plant. For qPCR quantification, three plants were surface-disinfected and three others were not to quantify epiphytic and endophytic bacterial colonization. Roots, shoots, and leaves were separated, weighed, and crushed in PBS. Samples of each plant organ were then diluted up to 10^{-6} and 100 µl from each dilution was spread in triplicate on LBA supplemented with spectinomycin (10 mg $liter^{-1}$) and incubated at 28°C for 24 h. The experiment was repeated twice.

Localization of mCherry-labeled strains in tomato with confocal laser scanning microscopy analysis. In parallel, plant organs were collected and the roots, shoots, and leaves were transversally/vertically cut and distributed on a microscope slide

with Fluoromount-G solution (Thermo Fisher Scientific). No surface disinfection was done for this observation. Ten-day seedling roots were also immersed for 3 h in a bacterial solution of C1M8::*mCherry* and C1M39::*mCherry* adjusted in PBS at 10^8 CFU ml^{-1} to determine whether the bacterial strains isolated from the seeds were able to move through the plant vascular tissues.

Observations were performed using a Leica Stellaris 8 Falcon confocal laser scanning microscope with the objective HC PL APO CS2 63×/1.20 water following collection at the UCLouvain IMABIOL platform. For the *mCherry* signal, the red emission was stimulated at 565 nm.

Statistical analysis

Statistical analysis was performed using RStudio version 4.3.0. The effect of bacterial coating on the seed vigor index was assessed for statistical significance between the treated groups and the control group using Student's *t* test ($P \leq 0.05$) after confirming the normality of residuals. For differences in the colonization of various plant organs by mCherry-tagged mutants, the Wilcoxon test was employed, as the residuals did not meet the normality assumption.

Results

Identification of seed-transmitted endophytic bacteria by metabarcoding

The sterility of the hydroponic culture system was confirmed, as no bacterial growth was observed on the medium inoculated with the hydroponic solution devoid of plants. Additionally, no amplification was detected following the 16S rRNA gene amplification in samples of the hydroponic solution without plants (Supplementary Fig. S5).

To capture the results of the colonization dynamics throughout plant development, from seeds to shoots and roots, metabarcoding-based analyses were performed using DNA extracted from surface-disinfected seeds, shoots, and roots of plants grown under sterile conditions. To ensure data robustness and reliability, replicate samples were used: three pools of seeds, six roots, and six shoots were collected. Metabarcoding was performed on 16S-ITS-23S amplicons, resulting in 682,539 high-quality duplex reads (BioProject PRJNA1136687) (Supplementary Fig. S6). After discarding chimeras and singletons, 675,222 reads remained, with an average abundance of 39,719 reads per sample and a standard deviation of 24,417 reads. The bioinformatics reconstruction of the bacterial communities identified a total of 53 distinct representative sequences. To simplify the discussion, we will talk about operational taxonomic units (OTUs).

A total of 13 OTUs were identified within the seeds, comprising the genera *Ralstonia*, *Bradyrhizobium*, *Sphingomonas*, *Paucibacter*, *Microbacterium*, *Stenotrophomonas*, *Lawsonella*, *Nocardioides*, and *Mucilaginibacter*. Of these, six genera, *Ralstonia*, *Bradyrhizobium*, *Sphingomonas*, *Paucibacter*, *Microbacterium*, and *Stenotrophomonas*, were ubiquitously present across all examined plant organs, indicating widespread distribution in the phytobiome from seeds (Figs. 2 and 3). These genera were defined as the core seed-transmitted endophytic bacterial community. By contrast, three genera, *Lawsonella*, *Nocardioides*, and *Mucilaginibacter*, were exclusively identified in the seeds, suggesting a unique ecological niche or specific environmental conditions favorable to these genera within the seed microhabitat. Additionally, 28 other genera were identified in roots and shoots, with 12 of these being common to both roots and shoots (Fig. 3).

The prevalent bacterial phylum identified in tomato was *Proteobacteria*, with *R. pickettii* being the most abundant species, comprising 98.9, 91.3, and 34.7% of the bacterial community in seeds, shoots, and roots, respectively (Fig. 2).

Comparison of the bacterial composition associated with seeds, shoots, and roots was performed using observed features as well as Shannon, Simpson, and inverse Simpson diversity indexes. This analysis revealed significant differences, with an increase in diversity in shoots and roots compared with seeds (Supplementary Fig. S7).

Beta diversity, analyzed with a Bray-Curtis distance matrix, underlined dissimilarity between the bacterial endophytic communities present in shoots, roots, and seeds, confirmed by permutational multivariate analysis of variance ($P = 0.0011$) (Supplementary Fig. S8). However, the observed dissimilarities were mainly due to rare OTUs. Indeed, 635,591 reads of bacterial endophytes were shared between seeds, roots, and shoots, representing 94.13% of total reads (93.25% of reads were shared in shoots, 93.06% were shared in roots, and 99.34% were shared in seeds).

In order to investigate the type (endophytic or epiphytic) of colonization of endophytic seed-borne bacteria, we compared the bacterial composition of plant organs with and without surface disinfection. For the roots, the genera *Luteolibacter*, *Paracoccus*, *Hymenobacter*, and *Moraxella* seemed to be epiphytic, as they were only found in nondisinfected roots. In parallel, a higher number of genera were epiphytes in shoots, including *Carnobacterium*, *Rhodococcus*, *Delftia*, *Flavobacterium*, *Luteitalea*, *Corynebacterium*, *Stenotrophomonas*, *Kineosporiaceae*, and *Brevundimonas* (Fig. 4).

The seed-transmitted core microbiota, composed of the genera *Ralstonia*, *Sphingomonas*, *Bradyrhizobium*, *Roseateles*, and *Microbacterium*, was found in roots and shoots regardless of surface disinfection status, confirming the endophytic nature. However, it is interesting to note that *Stenotrophomonas*, identified in seeds, was found to be an endophyte in roots but an epiphyte in shoots. Additionally, some genera were found to be endophytes only in one plant part, such as *Delftia*, *Brevundimonas*, *Cutibacterium*, *Achromobacter*, and *Rhodococcus* in roots and *Hymenobacter* and *Acinetobacter* in shoots. Surprisingly, eight bacterial genera were uniquely identified in surface-disinfected roots and another eight were identified exclusively in shoots (Fig. 4).

Identification of seed-transmitted endophytic bacteria by culture-dependent method

Four different nutrient-rich levels of medium were used during bacterial isolation to obtain taxonomically diverse strains of endophytes. In total, 111 bacterial strains were successfully isolated and purified, with 18 strains coming from seeds, 23 coming from roots, 40 coming from shoots, and 30 coming from the hydroponic solution of *S. lycopersicum* var. Moneymaker. The 16S rDNA gene sequencing enabled their classification into 23 genera from four phyla: *Firmicutes* (52.25%), *Actinobacteria* (25.26%), *Proteobacteria* (21.62%) (classes *Alphaproteobacteria* [9%], *Betaproteobacteria* [4.51%], and *Gammaproteobacteria* [8.11%]), and *Bacteroidetes* (0.87%). *Bacilli*, the most abundant class, was composed of the genera *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Rummeliibacillus*, *Peribacillus*, *Neobacillus*, *Aerococcus*, *Staphylococcus*, and *Fredinandcohnia*.

Figure 5 provides a summary of the isolated bacterial species and their origins. The highest abundance and diversity of species were observed in the shoots of the plant. The seeds predominantly contained bacterial endophytes from the *Firmicutes* phylum, constituting 94% of the total. In the shoots, endophytic bacteria were divided into four phyla, with *Actinobacteria* being the most prevalent (42.5%) followed by *Proteobacteria* (30%), including representatives of the *Alphaproteobacteria*, *Betaproteobacteria*, and

Gammaproteobacteria classes, and *Firmicutes* (25%) and *Bacteroidetes* (2.5%). The root-associated endophytes were primarily *Firmicutes* (60.9%), with *Proteobacteria* (21.7%) and *Actinobacteria* (17.4%) also represented. In the hydroponic solution, under sterile conditions, the endophytes likely originated from the seeds and roots, with the majority belonging to *Firmicutes* (56.7%) followed by *Proteobacteria* (23.3%) and *Actinobacteria* (20%).

RAPD analysis revealed that two isolates of *B. zanthoxyli* (C1M28 and C1M38) and two isolates of *B. centrosporus* (C1 25 and C1 26) were genetically and morphologically identical.

PGP traits of culturable seed-transmitted bacterial endophytes

Isolated tomato endophytes were first screened for their ability to tolerate osmotic stress. Among 111 isolates, 67 bacterial isolates were able to grow in LB supplemented with PEG 6000 (10% wt/vol) with a growth ratio between 0.5 and 1. Of the 67 bacterial strains selected (Fig. 5), 14 were identified as highly tolerant to drought, with an inhibition rate below 25%. Most of these belonged to the *Firmicutes* phylum (78.6%), including the species *B. velezensis*, *B. tequilensis*, *B safensis*, *B. zanthoxyli*, *P. frigoritolerans*, and *B. centrosporus*. In the phylum *Actinobacteria*, the species *M. aloeverae* displayed a high tolerance to osmotic stress, as did the species *P. protegens* in the phylum *Proteobacteria*. Twenty-six bacterial isolates, mostly within the *Firmicutes* phylum, had an inhibition rate between 25 and 50% and were considered drought tolerant (Fig. 5).

Among the 67 isolates, all but two produced IAA in the presence of the tryptophan precursor, with IAA production ranging from 0.32 to 15.65 µg ml^{-1}. Four bacterial isolates were identified as producing higher IAA content: *M. arabinogalactanolyticum* (C2 4), with 15.65 µg ml^{-1}; *B. tequilensis* (C2 20), with 10.72 µg ml^{-1}; *P. protegens* (C1M8), with 6.20 µg ml^{-1}; and *B. velezensis* (C1 45.1), with 6.12 µg ml^{-1}. Thirty of the 67 tested iso-

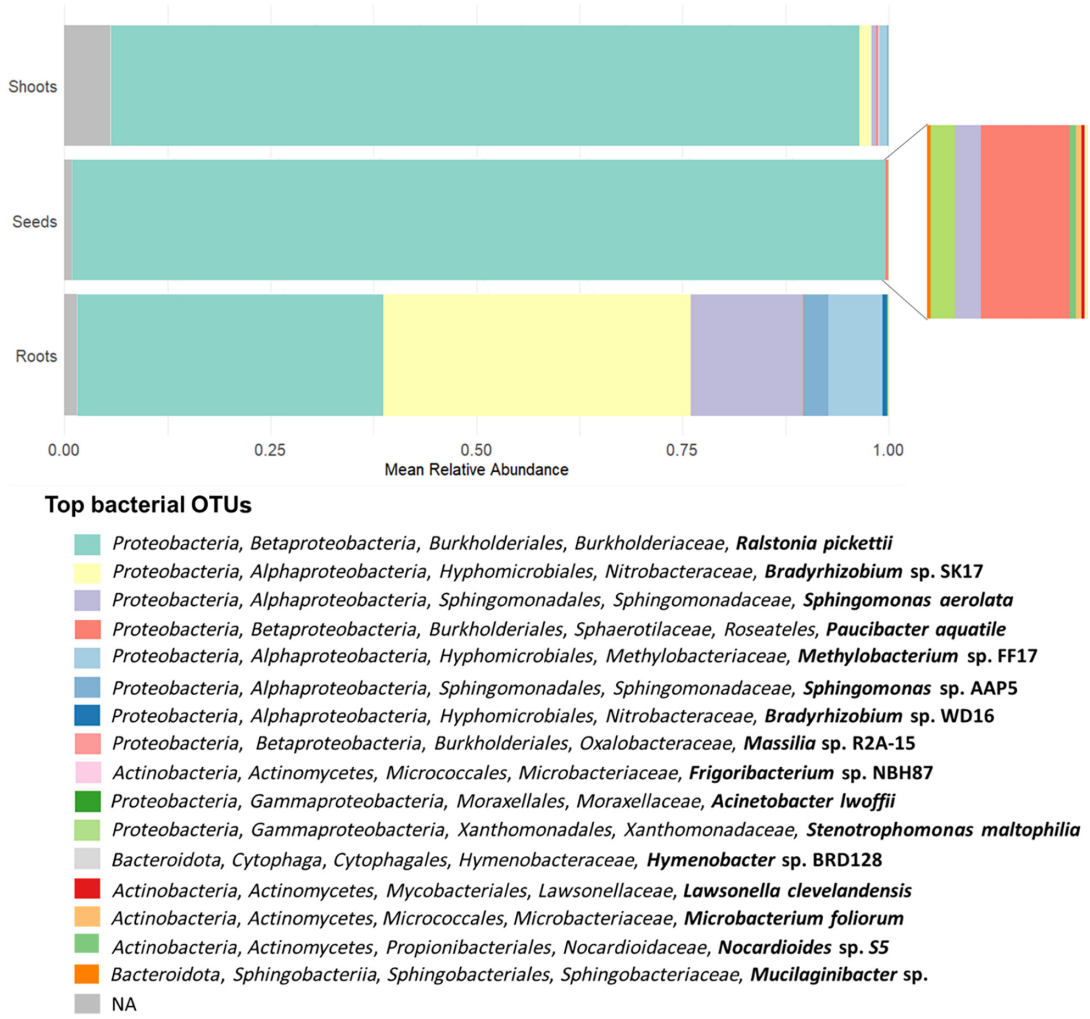

FIGURE 2
Composition of bacterial species in seeds, roots, and shoots of *Solanum lycopersicum* var. Moneymaker as detected by metabarcoding of the 16S-ITS-23S rRNA operon (*rrn*) (approximately 4,500 bp). To provide a comprehensive view of bacterial diversity, the results from both surface-disinfected and nondisinfected roots and shoots were merged. This approach was used to capture the entire bacterial community, including both epiphytic and endophytic species. None of the disinfected samples showed evidence of contamination, confirming that disinfection was effective. Three pools of tomato seeds, six shoots, and six roots were sequenced individually. Data were analyzed using PRONAME (Dubois et al. 2024), with high-quality reads clustered at a 90% sequence similarity threshold and singletons removed. Error correction involved polishing each centroid sequence with its corresponding subset of reads. Centroid reads were then processed with the BLASTN standalone tool and BLASTN results were filtered to retain only matches with an overall query coverage of 80% or higher, ensuring more consistent results with the online BLAST tool. NA = not applicable; OTUs = operational taxonomic units.

lates were shown to be able to solubilize phosphate in vitro, and most of these belonged to the genera *Bacillus*, *Paraburkholderia*, and *Pseudomonas*. Siderophore production ability was evidenced for 32 isolates, with higher production observed for bacteria belonging to the genera *Bacillus*, *Paraburkholderia*, and *Pseudomonas*. The production of EPSs was observed for 33 isolates in the genera *Bacillus*, *Peribacillus*, *Neobacillus*, *Rummeliibacillus*, *Brevibacillus*, *Microbacterium*, *Micro-*

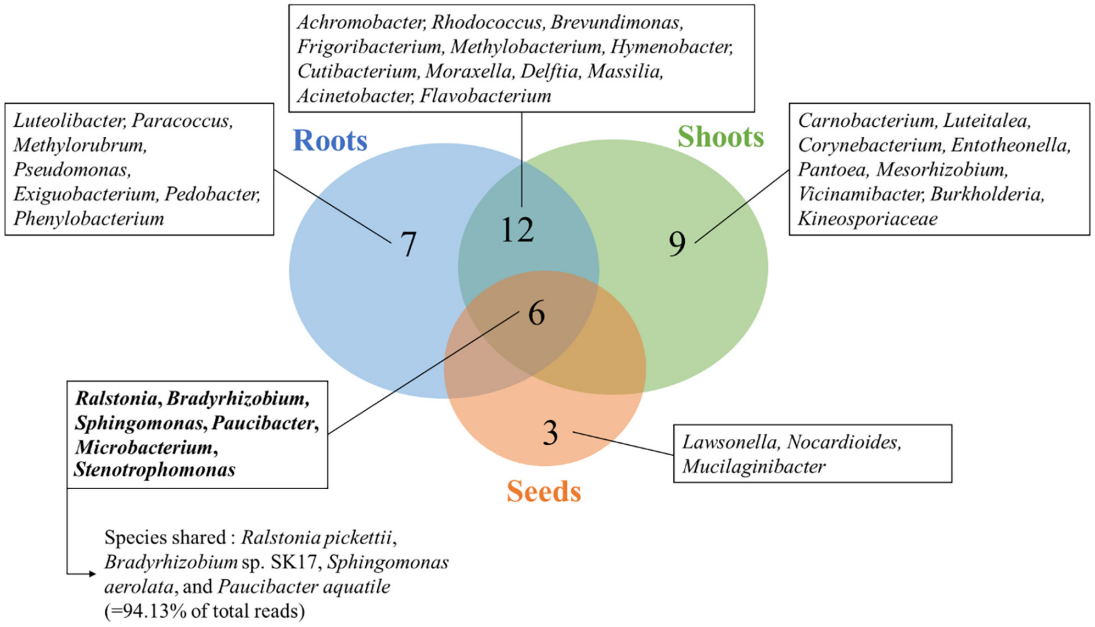

FIGURE 3
Venn diagram of vertically transmitted endophytic bacterial genera identified in tomato seeds and in roots and shoots of tomato grown in sterile conditions for 3 weeks. The percentage of reads common to all plant organs was 94.13%.

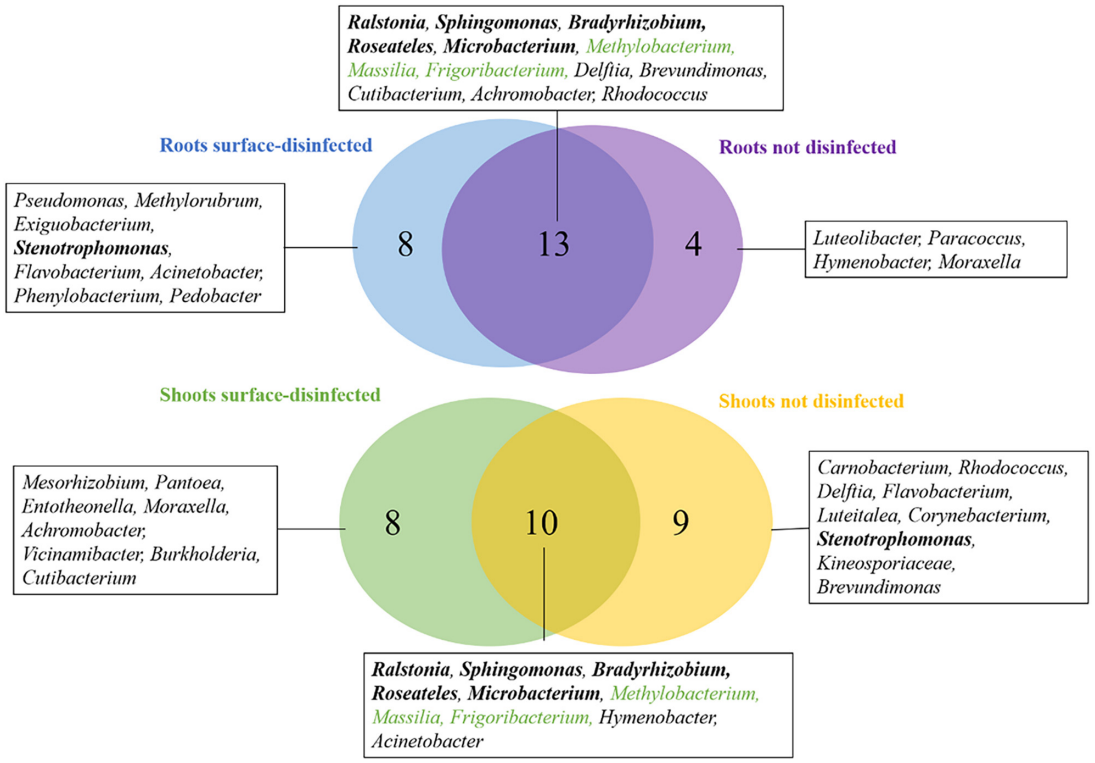

FIGURE 4
Venn diagram of vertically transmitted endophytic and epiphytic bacterial genera found in roots (blue and purple) and shoots (green and yellow) of tomato grown in sterile conditions for 3 weeks. Three pools of tomato seeds, three surface-disinfected shoots, three nondisinfected shoots, three surface-disinfected roots, and three nondisinfected roots were each sequenced individually. This approach was used to capture the entire bacterial community, including both epiphytic and endophytic species. None of the disinfected samples showed evidence of contamination, confirming that disinfection was effective. Genera shared between seeds, shoots, and roots are highlighted in bold. Genera shared between shoots and roots but not identified in seeds are in green.

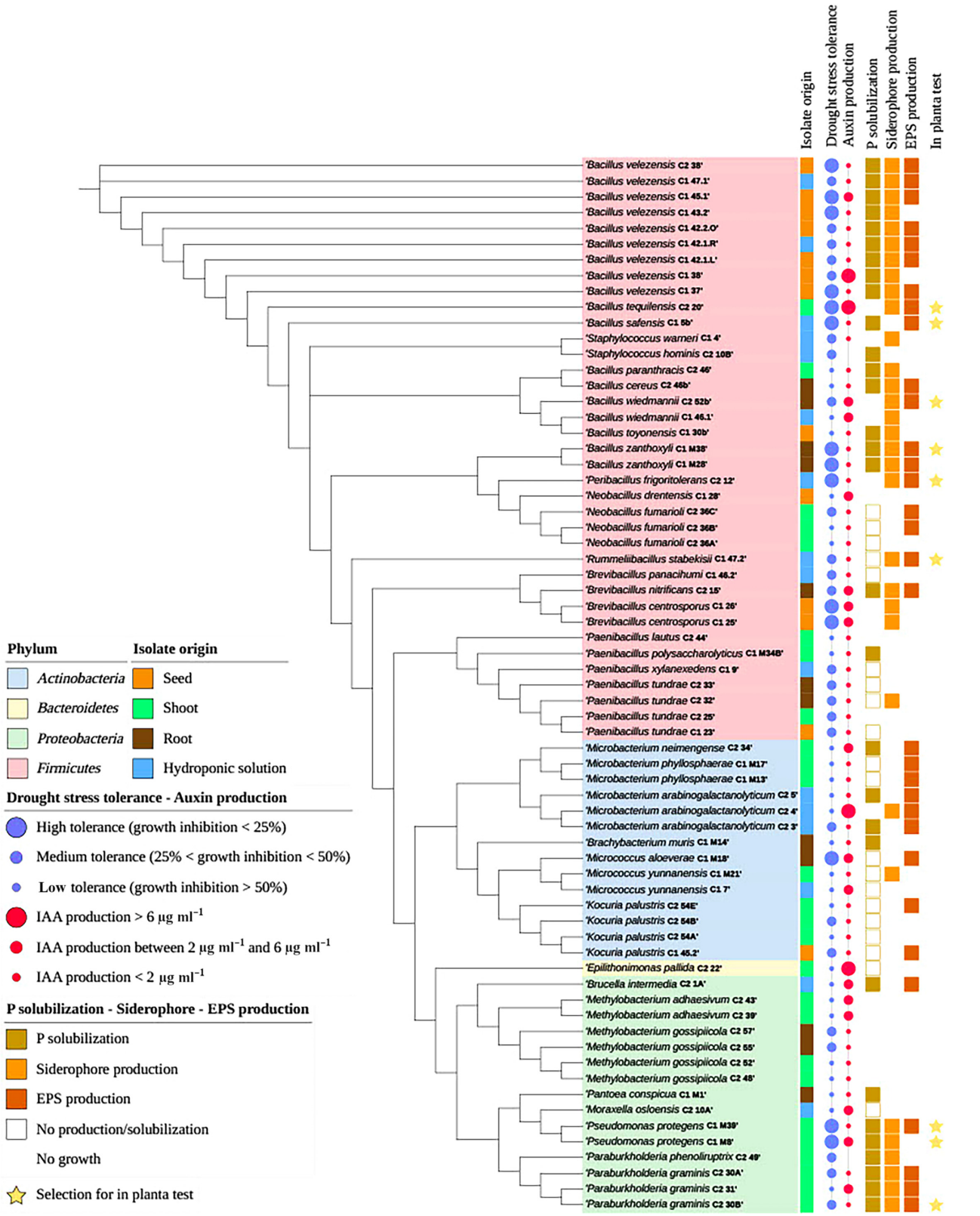

FIGURE 5

Phylogenetic tree of the cultivable endophytic bacterial strains of *Solanum lycopersicum* var. Moneymaker isolated from seeds, shoots, and roots and in hydroponic solution with summary of plant growth-promoting (PGP) traits. Circles are used for quantitative results. For drought stress, small, medium, and large circles represent bacterial strains with no or low tolerance (growth inhibition >50%), medium tolerance (growth inhibition >25 to <50%), and high tolerance (growth inhibition <25%) to osmotic stress (polyethylene glycol [PEG] 10% wt/vol), respectively. For auxin production, small, medium, and large circles represent indole-3-acetic acid (IAA) production of <2 µg ml^{-1}, 2 to 6 µg ml^{-1}, and >6 µg ml^{-1}, respectively. Phosphate (P) solubilization and siderophore and exopolysaccharide (EPS) production are represented as qualitative results. No square indicates that the bacterial strain does not grow on the selected medium and empty and solid squares indicate negative and positive results, respectively. Bacterial strains selected for in planta tests are marked with a star.

coccus, *Kocuria*, *Brucella*, *Pseudomonas*, and *Paraburkholderia* (Fig. 5).

After PGP tests in vitro, a selection was made for the in planta test. The main selection criteria included, first, a high resistance to osmotic stress and the production of EPSs and, second, the ability to produce auxin, solubilize phosphate, and synthesize siderophores. When several strains belonged to the same species, only one was selected. However, an exception was made for the genus *Pseudomonas*, for which two strains were chosen because of their distinct PGP characteristics and because the 16S rRNA identification method offers poorer resolution within this genus (Lauritsen et al. 2021). Initially, one strain of *B. velezensis* was selected, but this was later discarded because of difficulties with liquid culture. The nine bacterial strains selected were *B. safensis* (C1 5b), *P. frigoritolerans* (C2 12), *B. wiedmannii* (C2 52b), *P. graminis* (C2 30b), *B. zanthoxyli* (C1M38), *P. protegens* (C1M8 and C1M39), *R. stabekisii* (C1 47.2), and *B. tequilensis* (C2 20) (Fig. 5).

Effects of bacterial strains on seed germination and seedling growth under osmotic stress conditions

Seed coating with three of the nine selected bacterial strains led to significant enhancement of the seedling vigor index, obtained through germination percentage and shoot and root lengths (Supplementary Tables S9 and S10). Seeds coated with bacterial strains *R. stabekisii* (C1 47.2) and *P. frigoritolerans* (C2 12) led to more vigorous seedlings under both nonstress (0 MPa) and osmotic stress (−0.2 MPa) conditions. Conversely, seed coating with *P. protegens* (C1M8) exhibited an increased seedling vigor index solely under nonstress conditions (Fig. 6).

Confirmation of the endophytic behavior of isolated bacteria with mCherry mutants

The two *Pseudomonas* strains, C1M8 and C1M39, were selected for transformation because of the relative ease of creating mutants in gram-negative bacteria and because this genus was identified in both metabarcoding and medium isolation. The mCherry-tagged *P. protegens* C1M8 and C1M39 strains exhibited growth patterns and cell morphologies similar to the corresponding wild-type strains on LBA without spectinomycin. However, they fluoresced when exposed to red light. The insertion of the plasmid in these strains was confirmed by PCR. Using the confocal microscope, we determined that the cells from both strains (C1M8 and C1M39) remained alive and exhibited fluorescence in the free-living state (Supplementary Fig. S9).

Ten days after the germination of coated seeds, the mCherry-marked C1M8 and C1M39 strains were quantified in different plant organ tissues by colony counting and qPCR. During germination, bacterial strains proliferated and established colonization in both internal tissues and surfaces of roots and shoots. This primarily involved endophytic colonization, as no significant difference was observed in colony counts between surface-disinfected and nondisinfected plants for strains C1M8 and C1M39 ($P > 0.05$). Notably, both bacterial strains demonstrated similar colonization patterns in plant organs ($P > 0.05$), with a trend favoring an endophytic presence within the leaves followed by the roots and the shoots (Fig. 7), although without statistical significance (Supplementary Tables S11 and S12). Specifically, strain C1M8 exhibited heightened abundance within the leaves, registering concentrations of 1.71×10^{10} CFU per gram of fresh weight in nondisinfected plants and 1.67×10^{7} CFU per gram of fresh weight in disinfected plants. Conversely, strain C1M39 appeared to predominantly colonize the surface of roots and stems, with respective concentrations of 1.19×10^{10} and 4.46×10^{9} CFU per gram of fresh weight (Fig. 7; Supplementary Table S11).

The qPCR assay was performed in addition to the colony counting and confocal laser scanning microscopy analysis (Fig. 7) in the endophytic and epiphytic colonization process. The primers used in the TN7 qPCR showed an efficiency of 0.997 based on the slope and the line equation of the standard curve (slope = −3.328, $R^2 = 0.99$) (Supplementary Fig. S10), and no match to the plant DNA content (genome, plastid, and mitochondria of *S. lycopersicum*) was observed in Primer-BLAST analysis. No difference was observed in the colonization pattern of either bacterial strain or the colonization of plant organs by either bacterial strain, confirming the results observed by colony counting (Fig. 7).

The localization of mCherry-marked *P. protegens* C1M39 and C1M8 within the plant tissues was clarified by confocal laser scanning microscopy. Ten days after seed coating, the bacteria were primarily located in the apoplast spaces of all plant organs (Fig. 8B and C). Interestingly, the mCherry-marked C1M39 was observed inside a stoma, as highlighted in Figure 8C. After roots were immersed in the bacterial solution for 3 h, the presence of

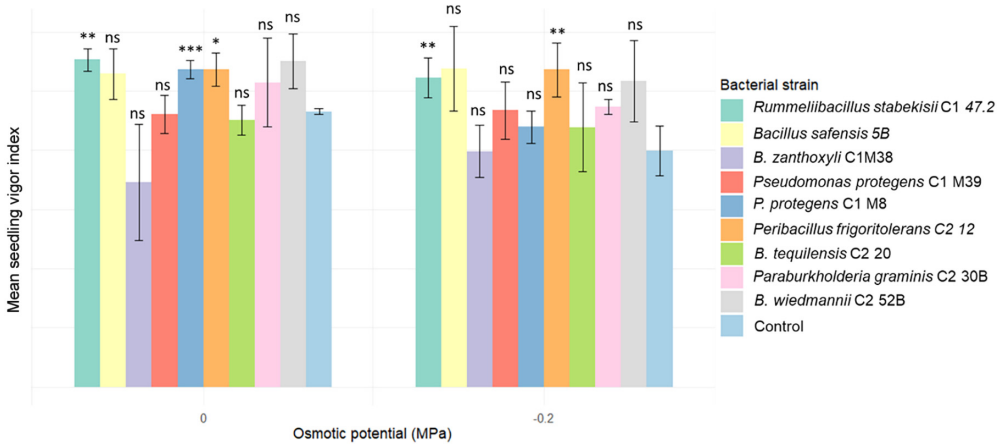

FIGURE 6
Seedling vigor index of tomato seed coating with bacterial strains under nonstress (0 MPa) and osmotic stress (−0.2 MPa) conditions (10 days after sowing). The significance of the data was assessed using a Student's *t* test, where ns represents $P > 0.05$, * represents $P < 0.05$; ** represents $0.01 < P < 0.05$; and *** represents $P < 0.01$ when comparing the vigor index of seedlings from coated seeds with the control group (phosphate-buffered saline [PBS]-coated seeds) ($n = 36$).

both bacterial strains was visible in vascular tissues, demonstrating the endophytic character of these bacteria (Fig. 8A and F). As observed in Figure 8E, bacteria penetrated the apical regions of roots to enter the plant vascular tissues (Fig. 8F) as well as the apoplast (Fig. 8D) and symplast (Fig. 8C).

Discussion

In this experiment, we deepened our knowledge regarding the seed-transmitted bacterial endophytic communities of *S. lycopersicum* var. Moneymaker using a sterile hydroponic culture sys-

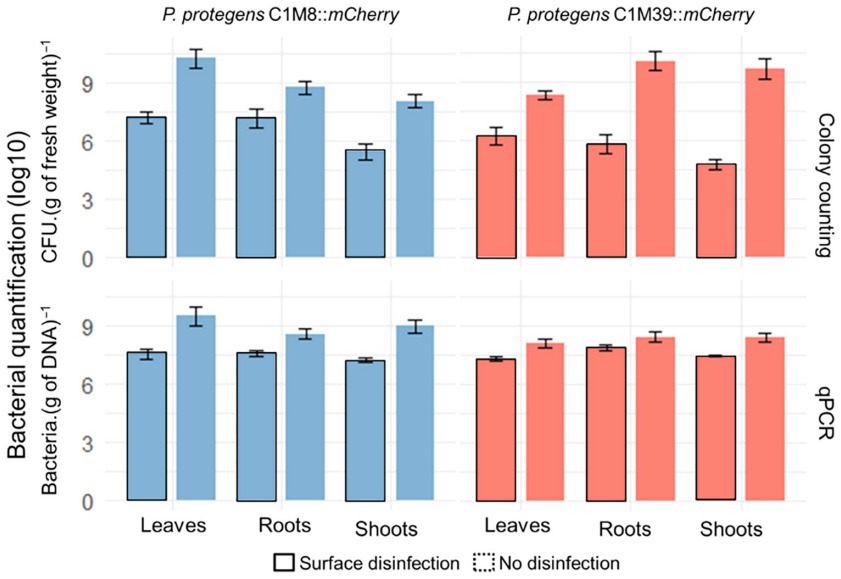

FIGURE 7
Quantification of mCherry-marked *Pseudomonas protegens* C1M8 and C1M39 with colony counting on medium ($n = 6$) and quantitative polymerase chain reaction (qPCR) ($n = 3$) in roots, shoots, and leaves of nondisinfected and disinfected tomato plants.

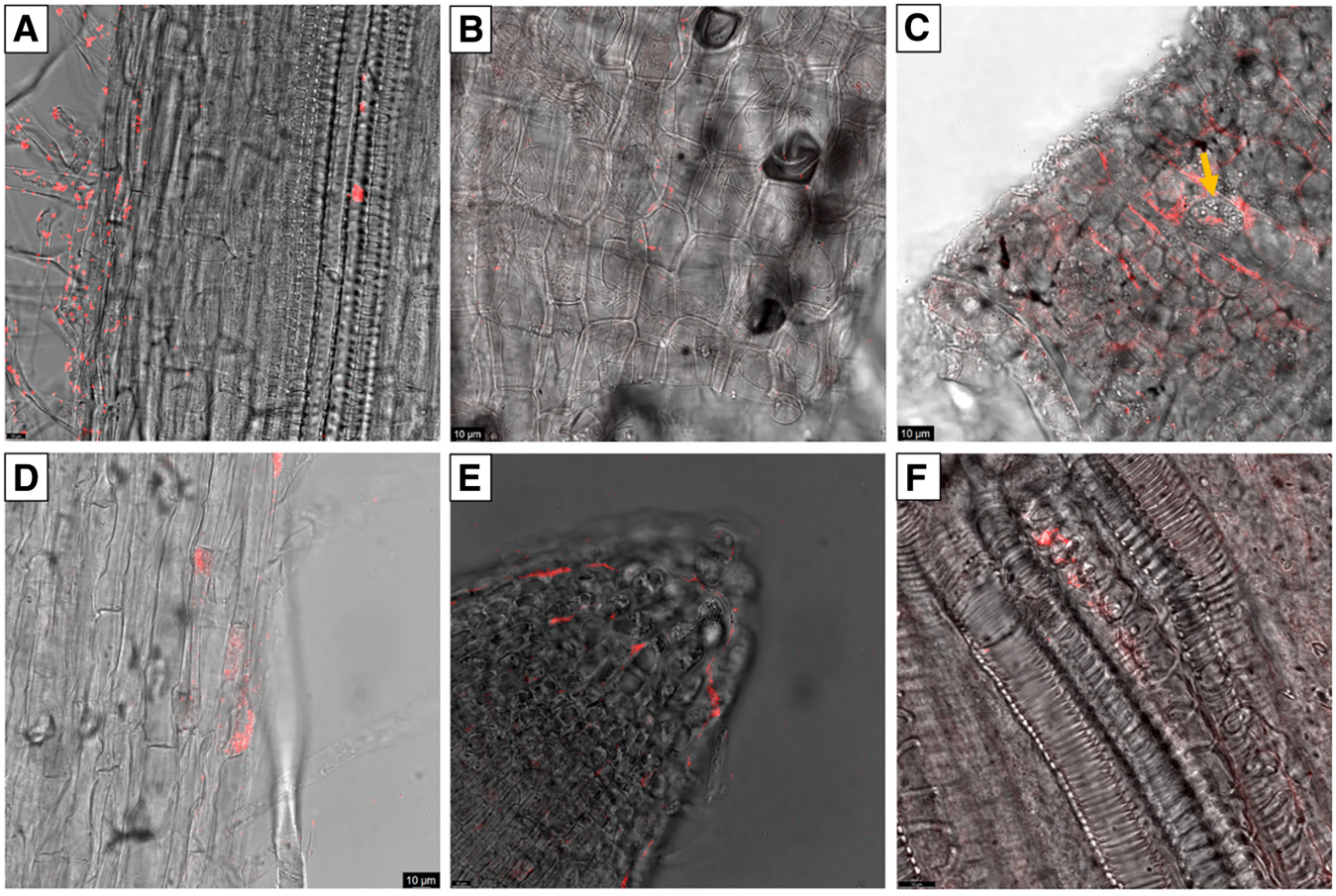

FIGURE 8
Confocal laser scanning microscopy analysis showing *Pseudomonas protegens* **A to C,** C1M39::*mCherry* and **D to F,** C1M8::*mCherry* in plants. A, Roots of 10-day seedlings that were immersed in C1M39::*mCherry* suspension adjusted to 10^8 CFU/ml for 3 h. B and C, roots and leaves of tomato seedlings grown under sterile conditions for 10 days from seeds coated with C1M39::*mCherry*, with C, arrow indicating stoma. D to F, Roots of 10-day plantlets that were immersed in C1M8::*mCherry* suspension adjusted to 10^8 CFU/ml for 3 h. D and E, Roots and apical roots, respectively. F, Shoots of tomato after root immersion.

tem in which plants were grown for 3 weeks. The communities were characterized through a polyphasic approach, combining metabarcoding and culture-based methods.

Key species in tomato plant bacteriome and colonization specificity

To accurately characterize the endophytic bacteria using metabarcoding, it was crucial to minimize bias in tomato sequence amplifications caused by chloroplast and mitochondrial DNA while maximizing the recovery of reads from endophytic bacteria (Fitzpatrick et al. 2018b). We addressed this challenge by designing a specialized PNA clamp tailored to the tomato variety Moneymaker, which significantly reduced the proportion of tomato reads from 60.2 to 0.14% of the total reads. By combining this advanced method with sequencing of the 16S-ITS-23S region of the *rrn*, we achieved species-level identification of endophytic communities within seeds, roots, and shoots. Alpha diversity indexes of endophytic bacterial communities between seeds, shoots, and roots (Supplementary Fig. S7) highlighted that the core microbiota increased upon seed germination in roots and shoots. This result suggests that rare bacterial species present in small relative abundance in seeds were able to thrive, increasing their relative abundance (Abdelfattah et al. 2021; Faddetta et al. 2021).

To capture the most comprehensive picture of microbial transfer from seed to plant tissues, we compared the microbial communities of seeds, roots, and shoots by analyzing surface-disinfected and nondisinfected samples for both roots and aerial parts. This approach allowed us to examine the entire bacterial community transferred from the seed to the plant tissues (as seen in nondisinfected samples) while also detecting endophytic species that may not have been detectable in the presence of the abundant seed-borne epiphytic bacteria. This is crucial because bacteria that are endophytic within seeds may become epiphytic on aerial parts or roots (Hardoim et al. 2012). However, many studies on the transmission of endophytic bacteria from seeds often overlook these diverse colonization modes (Bergna et al. 2018; Faddetta et al. 2021).

The differences observed in beta diversity (Supplementary Fig. S8) can be partly explained by this approach, as the microbial communities in surface-disinfected roots displayed notable differences compared with nondisinfected roots. For instance, *R. pickettii* was significantly reduced in abundance in surface-disinfected roots, suggesting that it primarily resides on the root surface rather than as an endophyte. By contrast, taxa such as *Bradyrhizobium* and *S. aerolata* increased in relative abundance in the absence of *Ralstonia*, indicating that the reduction in surface-associated bacteria can reveal a clearer view of less abundant endophytic species. Regardless of whether these distinctions are plant-mediated or microbe-mediated, this finding aligns with studies showing that microbial community structures in aboveground and belowground plant parts can differ substantially, originating during the transmission from seed to seedling (Kong et al. 2019).

Additionally, we observed niche differentiation in the colonization patterns of bacteria such as *Stenotrophomonas*, which displayed endophytic colonization in roots and epiphytic colonization in shoots. These variations may result from a combination of microbial life history traits and plant regulatory factors that guide bacteria to specific plant parts (Abdelfattah et al. 2021). The development of these species, present in low relative abundance in seeds, within the root endosphere may explain the observed differences in beta diversity (Supplementary Fig. S8). Notably, OTUs shared across seeds, shoots, and roots—when shoots and roots were considered without accounting for disinfection—represented 94% of the total reads.

Intriguingly, certain taxa observed in low quantities in the shoots and roots of the developing seedlings were not initially detected in the seeds. This observation was noted in the work of Faddetta et al. (2021), which demonstrated increased microbial diversity in the shoots of 15-day-old *Citrus limon* plantlets grown under sterile conditions on Murashige and Skoog medium, including the detection of new genera absent from the original seed communities. Similarly, Abdelfattah et al. (2021) documented the transmission of endophytes from seeds to shoots and roots of oak seedlings grown in a microbe-free environment, with some taxa identified in the phyllosphere and roots that were not initially detected in the seeds. Huang et al. (2017) further supported this concept by identifying bacterial and fungal OTUs in peanut sprouts that were absent in the cotyledons and germs, suggesting the emergence of certain microbial species after germination.

This discrepancy can be explained by several interrelated factors. Biologically and statistically, variability among individual seeds might lead to differences in the microbial communities present initially in the seeds compared with those emerging in the seedlings. Additionally, it is possible that some taxa, initially undetectable, could have proliferated after being transmitted to the growing seedlings. Furthermore, dominant genera, such as *Ralstonia* in this study, might obscure the detection of less abundant taxa. This hypothesis is supported by the fact that surface disinfection enables the detection of genera that are not observable without such treatment. Finally, although the possibility of minor experimental contamination cannot be entirely dismissed, the sterility of the system was thoroughly validated, making contamination an unlikely explanation.

Our research focused on characterizing the core microbiome, specifically identifying bacterial species that consistently appear in both the seed and the seedling. Given the high percentage of shared reads between plant organs, our findings likely represent the core microbiota transmitted from the seeds to the roots and shoots, including *R. pickettii*, *Bradyrhizobium* sp. SK17, *S. aerolata*, and *P. aquatile*. Interestingly, *Ralstonia* and *Sphingomonas* were also central to the seed-borne microbiota of the tomato cultivars Arka Vikas and Arka Abha, suggesting that certain genera may be common to the core microbiota regardless of the cultivar or the experimental conditions (Shaik and Thomas 2019) . *R. pickettii*, present in high abundance in all plant organs, is an oligotroph, thriving in nutrient-scarce environments, and various strains of *Ralstonia* are resistant to stress caused by glyphosate herbicides or heavy metals such as manganese (Huang et al. 2018; Kuklinsky-Sobral et al. 2005). Additionally, another strain from the tomato rhizosphere was shown to act as a biocontrol agent, offering protection against *R. solanacearum* (Wei et al. 2013). The presence of nitrogen-fixing *Bradyrhizobium* in the primary microbiota of hydroponically grown tomato plants aligns with expectations given its diazotrophic endophytic properties (Terakado-Tonooka et al. 2023). These characteristics are especially beneficial in hydroponic systems with controlled oxygen levels that favor nitrogen fixers (Smercina et al. 2019). Other studies revealed that the core microbiota of tomato generally comprises a predominance of *Proteobacteria* as well as *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* (López et al. 2020; Tian et al. 2017).

The near-complete absence of *Firmicutes* in our experiment analyzing the seed-transmitted endophytic bacteria through sequencing is particularly unexpected given the results of the culture-dependent analysis and the fact that *Bacillus* spp. are considered the most common seed-associated bacteria across plant species (Nelson 2018; Truyens et al. 2014). The nonoc-

currence of the *Bacillus* genus may be attributed to the analysis method. Shaik and Thomas (2019) showed a predominance of *Firmicutes* in culture-based methods, whereas *Proteobacteria* were more dominant in metabarcoding studies (65.7 to 69.6% OTUs). Additionally, it is crucial to acknowledge that the methodological approach employed in DNA extraction and sequencing could significantly influence our ability to detect *Bacillus* spp. Gram-positive bacteria have particularly robust cell walls that can hinder effective DNA extraction, and their propensity to secrete DNases may degrade DNA, complicating its sequencing. Indeed, our reliance on longer amplicons (exceeding 4 kb) for sequencing may pose challenges in detecting partially degraded DNA. This phenomenon was notably observed in a recent study in which *Bacillus* was detectable using Illumina technology but was missed in Nanopore sequencing using two different sets of primers targeting the full operon (B. Dubois, *unpublished data*). This hypothesis was supported by our study of cultivable seed-transmitted endophytic bacteria, which revealed bacterial isolates predominantly belonging to the phyla *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. This result is consistent with those typically reported for cultivable endophytes in the literature (Truyens et al. 2014).

Comparison of endophytic communities identified through metabarcoding and culture-dependent methods

When comparing culture-dependent and metabarcoding methods to study seed-transmitted endophytic bacteria, we observed notable differences in the results. Such discrepancies are commonly observed in similar studies and can be attributed to several factors (Faddetta et al. 2021; Zeyaullah et al. 2009). One key reason is that culture-dependent methods identify only culturable bacteria, whereas metabarcoding may miss bacteria present in very small quantities that can still be isolated through cultivation. Additionally, the medium selected for bacterial isolation, such as extraction and amplification processes used in metabarcoding, can introduce biases, affecting the bacterial species detected. A significant example in our study is the prevalence of *Bacillus* (phylum *Firmicutes*) in the culture-dependent method, accounting for 78.6% of isolates, whereas this genus was largely absent in the metabarcoding analysis. This is one of the main reasons behind the differences between the two approaches, likely due to the difficulty in amplifying *Bacillus* through metabarcoding techniques, as explained in the section "Key species in tomato plant bacteriome and colonization specificity." Another could be linked to bias introduced by the amplification of the 16S-ITS-23S *rrn* (Hugerth and Andersson 2017). However, this is unlikely, as the primers were validated against three other commonly used primer sets and confirmed to be universal.

Despite the species-level information provided by metabarcoding, identification of cultivable isolates was done through 16S Sanger sequencing, which does not allow resolution at the species level for every genus, specifically for *Pseudomonas*. This is why we made a comparison at the genus level for the first time and observed that 20 isolates matched genera identified through metabarcoding, underscoring the need to use both approaches to obtain a more complete understanding of the microbial community and its diversity.

Among the isolates selected for the in planta test, *P. frigoritolerans* (strain C2 12), which enhances tomato seedling vigor under both stress and nonstress conditions, was also identified in the metabarcoding study.

PGP bacteria among seed-transmitted bacteria

The culture-dependent approach allowed us to explore the properties and functions of a significant portion of the seed microbiota. This functional aspect is crucial, as it provides insights into the roles these endophytes play in plant health and development, emphasizing their importance beyond their mere presence within the plant. Our results underscore the importance of seed-transmitted bacteria in enhancing plant tolerance to abiotic stress. Most isolates were capable of producing IAA and solubilizing phosphate, whereas approximately half could produce siderophores and EPSs. These functions can facilitate plant growth directly, indirectly, or synergistically (Cherif et al. 2015; Rolli et al. 2015; Vurukonda et al. 2016). For instance, bacterial auxin production has been shown to enhance plant yield under drought stress (Raheem et al. 2018), playing a critical role in regulating plant responses to salinity and drought by influencing gene expression through auxin response factors, which regulate soluble sugar content, promote root development, and maintain chlorophyll levels, helping plants adapt to these environmental stresses (Verma et al. 2022). Furthermore, phosphate solubilization can mitigate the negative effects of drought by increasing root biomass and enhancing root hydraulic conductance under drought conditions (Abbasi 2023). Siderophore-producing microbes alleviate iron deficiency and improve the physiological and biochemical processes of crops in saline soils, which are similar to osmotic stress conditions (Singh et al. 2022). Additionally, EPS-producing bacteria help maintain higher soil moisture content, protect plant roots from desiccation, and facilitate microbial aggregation, thereby enhancing plant–microbe interactions (Naseem et al. 2018). Notably, more than half of the isolates were able to grow under osmotic stress (10% PEG), suggesting that these bacteria may possess traits that allow them to remain active in stress conditions, such as those encountered within or on seeds, which could select for osmotic stress tolerance (Chen et al. 2017). However, although this demonstrates the ability of isolates to thrive in stressful environments, it does not necessarily indicate that they confer drought tolerance to the seedlings. Indeed, the expression of these PGP traits in vitro does not guarantee their effectiveness in planta, where community competition and environmental complexity may affect bacterial function (Hossain et al. 2023).

Seed coating trials further confirmed the value of several seed-transmitted endophytic bacteria. Selected strains (*P. frigoritolerans* C2 12 and *R. stabekisii* C1 47.2) enhanced root and shoot growth under both stress and nonstress conditions, increasing plant vigor. These strains demonstrated osmotic stress tolerance and significant EPS and siderophore production in PGP tests. Additionally, *P. protegens* C1M8, which can solubilize phosphate and produce siderophores, enhanced plant vigor under only nonstress conditions, likely because of its lack of EPS production, a key factor in plant drought resilience (Naseem et al. 2018). These findings highlight that the PGP activity of bacteria can be either stress-dependent or stress-independent (Rolli et al. 2015). Further supporting our findings, previous studies have highlighted the beneficial roles of similar strains. *P. frigoritolerans* T7-IITJ has been reported to induce PGP genes in *Arabidopsis thaliana* under osmotic stress (Marik et al. 2024), and *P. frigoritolerans* A70 has been shown to enhance the performance of *Medicago polymorpha* under osmotic stress (Gil et al. 2023). Similarly, *R. stabekisii* is known as a halotolerant species with biomineralization potential (Mudgil et al. 2018).

Colonization abilities of seed-transmitted endophytic bacteria

In addition to their PGP traits, efficient colonization by inoculated bacteria is a crucial step in plant–endophyte interactions (Compant et al. 2005). The colonization ability of *P. protegens* C1M8::*mCherry* and C1M39::*mCherry* mutants was confirmed when applied through seed coating or root dipping. When

seeds were coated, the bacteria were predominantly observed in apoplast spaces rich in nutrients (Hardoim et al. 2015) but not plant vessels. On mature ungerminated seeds, bacteria colonized the seed coat (Barret et al. 2016), suggesting migration with plant development rather than through vessels. Conversely, root dipping led to colonization on the root surface, penetrating at root tips and lateral root cracks and spreading to the apoplast, symplast, and vascular tissue. Bacteria were observed in shoots within 3 h, confirming propagation through vascular tissues, similar to *Burkholderia phytofirmans* PsJN in grapevine plantlets (Compant et al. 2005). The mode of inoculation influenced colonization patterns: seed coating restricted bacteria to specific zones, whereas root dipping allowed extensive xylem colonization because of increased entry points. This extensive colonization permits rapid, passive bacterial propagation without degrading enzymes, as shown with *B. phytofirmans* strain PsJN in *Vitis vinifera* L. (Compant et al. 2008). These findings highlight the adaptability of these strains in plant colonization. Furthermore, their presence in the stomata and around the trichomes may raise further questions about their impact on plant physiology.

To conclude, this study, utilizing a combination of culture-dependent methods, metabarcoding, and confocal analysis, underscores not only the diverse range of seed-transmitted endophytic bacteria but also their dynamic nature and significant potential to influence plant growth under various conditions. Notably, major differences were observed between the microbiome profiles obtained through DNA-based and culture-dependent methods, particularly for *Firmicutes*, which were prevalent in culture but nearly absent in the metabarcoding results. These findings highlight the limitations of each method in capturing the full microbial community and the adaptive capabilities of endophytes within ecological niches.

Looking ahead, key questions about the longevity of these interactions persist, particularly whether older seeds are more susceptible to losing these beneficial partnerships, which could impact germination and seedling vigor. Further research is needed to determine the transmission of these bacterial communities to subsequent generations and assess the long-term stability and evolutionary implications of these interactions. Exploring the influence of these microorganisms on the floral microbiome could provide insights into their effects on plant reproduction and health. Additionally, studying variations across different cultivars could reveal the genetic or environmental factors that influence microbial community composition and functionality. The interplay between endophytes and external microbial populations also warrants investigation, as it could crucially affect the resilience and adaptability of the core microbiota.

From a practical point of view, exploiting specific compositions of the core microbiota to adapt microbial treatments could help sustainable agricultural practices, improving crop resistance, yield, and health. Future research should also aim to compare metabarcoding and functional analyses to deepen our understanding of these interactions and their practical applications in agriculture.

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