Impact of bacterial inoculations on *Pisum sativum* L. performance and associated bacterial and AMF communities under semi-arid field conditions in Tunisia

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Abstract

In Mediterranean agroecosystems, pea (*Pisum sativum* L.) is one important crop due to its nutritional benefits and high protein content. However, soil nutrient availability and soil health are known to affect pea productivity, especially under arid and semi-arid conditions. Currently, the use of plant growth-promoting bacteria (PGPB) may represent a bio-based tool to improve pea productivity in drought-affected areas. Nevertheless, there is limited knowledge on how PGPB inoculations in field could impact native communities of bacteria and arbuscular mycorrhizal fungi (AMF) in these areas. Here, a two-year field study in Tunisia was established to evaluate the effects of inoculating two pea varieties with three strains of potential PGPB, including *Rhizobium laguerreae* and two strains of *Erwinia* sp., on agronomic performance and soil microbial communities. Inoculations improved productivity and all measured agronomic parameters, with the treatment including a consortia of the three strains showing the highest benefits. Metabarcoding analysis showed an increased bacterial and AM fungal diversity in soil of inoculated plants. Additionally, specific AMF-bacterial associations were identified, suggesting a synergistic role in enhancing soil health and pea growth. Overall, this study highlights the potential of targeted bacterial inoculations to improve pea performance under semi-arid environments by exploiting beneficial plant-microbe interactions. These results support the use of microbial inoculants as a sustainable agricultural practice in semi-arid areas, also improving the understanding of their impact on native bacterial and AM fungal communities.

Keywords

Pea, arbuscular mycorrhizal fungi, PGPB, plant-microbe interactions

1. Introduction

In Mediterranean region, leguminous species are a key component of agroecosystems (Maxted and Bennett, 2001) and, among them, pea (*Pisum sativum* L.) represents one of the most cultivated (Missbah El Idrissi et al., 2020). Pea, known for its substantial nutritional and health benefits (Ilahi et al., 2021), is an important leguminous crop especially valued for its high protein content in developing countries (Lu et al., 2019; Ejaz et al., 2020). Additionally, it is considered a functional food due to the presence of bioactive compounds like phenolic ones, which have health-promoting properties, *i.e.*, antioxidant and anti-inflammatory (Nazir et al., 2020). However, the availability of nitrogen (N) and phosphorus (P) nutrients is crucial for the accumulation of phenolic compounds in plant tissues and plays an important role in determining the antioxidative status of pea plants (Stewart et al., 2001). Due to pea low tolerance to biotic and abiotic stress, major problems in its cultivation are represented by yield instability in relation to site and season variability (Rubiales et al., 2014). As a result, pea cultivation is particularly limited in the Mediterranean Basin and recently released pea cultivars are poorly adapted to Mediterranean environments (Rubiales et al., 2021).

Tunisia is currently considered as a climate change hotspot. In the agricultural systems the damages and losses for crops resulting from climatic issues are increasingly reported (Young et al., 2012). In the last years, the combined use of chemical fertilizers and drought have led to a decreased agricultural production (Radhouane, 2018; Besser et al., 2021). In this scenario, the need of the development and applications of sustainable alternatives to chemical fertilizers, as the use of microbial-based solutions, has been rising. Currently, in sustainable agricultural management, alternatives to the chemical fertilization by using environmental-friendly practices to enhance crop productivity are considered as promising tools (Yadav, 2020). Sustainable approaches include the use of bio-based solutions relying on the interactions between plants and beneficial microorganisms, including plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) (Hakim et al., 2021; Balestrini et al., 2024). These interactions play a crucial role in enhancing plant nutrient uptake and soil fertility, while also influencing the surrounding microbial communities in the rhizosphere (Trivedi et al., 2020).

Plant growth-promoting bacteria are a group of bacteria able to improve plant growth and fitness without establishing symbiotic relationship with the host roots (Mantelin and Touraine, 2004). It has been demonstrated that they can be used as biofertilizers to improve crop productivity and resilience, particularly under challenging environmental constraints such as salinity and aridity (Gritli et al., 2022; Ilahi et al., 2024). Members of PGPB are known to enhance biological nitrogen fixation (Mahmoud et al., 2023), thereby reducing reliance on nitrogen fertilizers and contributing

to soil health. PGPB are an heterogenous group of bacteria that include several different genera *e.g.*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, and *Variovorax (*Arora et al., 2016).

Among PGPB, strains of *Erwinia* sp. have proved to enhance crop growth and resistance to abiotic stresses (Ilahi et al., 2024) *via* different mechanisms *i.e*, nitrogen fixation, phosphate solubilization and siderophore production (Tozlu et al., 2012), as well as to improve plant tolerance to salt stress (Singh et al., 2018). Moreover, strains of *Erwinia* sp. are known to be able to improve plant tolerance to drought producing bacterial 1-aminocyclopropane-1-carboxylic acid (AAC)-deaminase, volatiles, antioxidant compounds, cytokinin and indole-3-acetic acid (IAA) (Kasotia et al., 2016), therefore representing a sustainable approach in arid and semi-arid environments to boost crop productivity.

Differentially from PGPB, rhizobia establish symbiotic relationship with plant through the formation of root nodules (Mantelin and Touraine, 2004). Rhizobia are a group of bacteria belonging to Alphaproteobacteria and Betaproteobacteria classes, they are able to produce N_2-fixing nodules (Poole et al., 2018), and they are known for their ability in establishing root symbiosis with leguminous plants *e.g.,* pea (Ejaz et al., 2020). Some rhizobia species identified as beneficial microorganism include *Rhizobium leguminosarum*, *R. pisi*, *R. fabae*, *R. laguerreae*, *R. bangladeshense*, *R. lentis*, *R. binae* and *R. anhuiense* (Ilahi et al., 2021). In particular *R. laguerreae* species have been identified as the most represented ones in Tunisian soils among *Rhizobium* species (Ilahi et al., 2021). It has been reported that biological nitrogen fixation, mediated by rhizobia (Lindström and Mousavi, 2020) can supply more than 50% of the required nitrogen to pea plants, contributing approximately 25 kg of nitrogen *per* hectare to the soil for the subsequent crop (Ejaz et al., 2020). Despite a slightly lower effectiveness compared to mineral N fertilization, the importance of selected rhizobium inoculation in increasing leguminous crop yields has been confirmed (dos Santos Sousa et al., 2022), suggesting their use as bio-based alternatives to inorganic fertilization.

Similarly to rhizobia, arbuscular mycorrhizal fungi (AMF) are symbiotic organisms able to establish strong relationships with plant roots, enhancing nutrient and water uptake (Pasquini et al., 2023). For this reason, they represent a promising tool in improving plant tolerance to water deficit conditions and lack of nutrients in the soil (George and Ray, 2023). Moreover, the symbiotic relationship between plant roots and AM fungal hyphae leads to the recruitment of specialized microorganisms in the so-called mycorrhizosphere, *i.e.,* the soil area influenced by roots colonized by AMF (Giovannini et al., 2020; Williams et al., 2024). This microbial recruitment has been

reported to play a positive role in plant development, nutrient absorption, as well as tolerance to abiotic and biotic stresses (Philippot et al., 2023). In arid and semi-arid regions, accelerated desertification has led to soil degradation and reduced vegetation cover, affecting plant growth (Mahmoudi et al., 2019). In these regions, symbiotic relationships between plants and AMF are vital for sustaining plant growth, particularly in protected Mediterranean conservation areas (Mahmoudi et al., 2020). Recent studies have been performed to explore AM fungal distribution in natural soils, by evaluating the relative abundances of different genera of virtual taxa (VTs) of AMF in arid and semi-arid environments (El Hazzat et al., 2018; Dalli et al., 2020; Gritli et al., 2023; Nooren et al., 2023). According to Gritli et al. (2023), most of the identified AM fungal genera, associated to *Lathyrus cicera* roots and soils in northern Tunisia, were represented by *Glomus*, *Claroideoglomus*, *Diversispora*, *Paraglomus*, and *Scutellospora*. In comparison, a study in western Algeria showed *Glomus* and *Acaulospora* as dominant genera associated with carob trees (Dalli et al., 2020). Moreover, *Glomus* has been detected as the most representative AM fungal genus in Morocco and Pakistan in chickpea, beans, and other legumes (El Hazzat et al., 2018; Nooren et al., 2023). Nevertheless, knowledge about natural plant-AM fungal interactions in arid ecosystems, including Tunisia, is still limited. Moreover, the influence of the use of bacterial inoculants in field on fungal communities has been proved to have various effects and no clear results have been proved (Trabelsi and Mhamdi, 2013).

The aim of this two-year field study was to evaluate the beneficial effects of inoculation with one strain of *Rhizobium laguerreae* (Ilahi et al., 2021) and two strains of *Erwinia* sp., previously characterized as PGPB (Ilahi et al., 2024), on the agronomic parameters and overall plant performance of two *P. sativum* varieties grown in fields in Tunisia. The second focus of this study was to examine the impact of the bacterial inoculations on pea-associated microbial taxa, focusing on soil AM fungal and bacterial communities. Reveling the variety dependent responses to microbial inoculations will allow to explore the potential of microbial inocula to enhance pea plant growth and productivity by exploiting the synergistic interactions between specific bacterial and AM fungal communities in the soil, thus promoting sustainable agriculture practices.

2. Materials and Methods

2.1 Experimental Design

The experiment was conducted at the experimental site of the Higher School of Agriculture of Mateur, located in the governorate of Bizerte, delegation of Mateur, approximately 70 km northwest

of Tunis. The site is located at a latitude of 37°3'7.017"N, longitude of 9°37'13.429"E and has an elevation of 19 meters above sea level. The experiment was conducted from December 1 to April 29, in 2021 and 2022, respectively. The used plant material consisted of two varieties of pea *(Pisum sativum* L.), namely the very early variety "Douce de Provence" (DP) and the early variety "Merveille de Kelvedon" (MK). The experimental design was a randomized complete block design, for a total area of 350 m², partitioned into two equal plots of 175 m² for each pea variety. These plots were delineated with a 2-meter margin to minimize cross-contamination and ensure distinct separation of the experimental factors (Fig. S1). Each plot was further divided into three sub-plots dedicated to each of the three different treatments: i) inoculation with a *Rhizobium* strain (Rh), ii) inoculation with *Rhizobium* and *Erwinia* strains (PG Rh) and iii) not-inoculated control (CTRL), for a total of nine sub-plots, each of 14 m² and arranged in eight rows spaced 0.5 meters apart (Fig. S1). The allocation of pea varieties in the plots was determined by their maturation rates: DP was planted in the first plot due to its earlier maturity, while MK was assigned to the second plot. The cultivation protocol initiated with land preparation, plowing to a depth of 20 cm followed by two subsequent passes with an offset implement, to ensure soil aeration and preparation. Planting rows were manually established, followed by the hand sowing of seeds at prescribed densities of 20 seeds/m² for *P. sativum.* Organic cultivation practices were employed, omitting chemical treatments or fertilizers, with manual weeding using a hoe. No irrigation practices were applied to pea plants, *i.e.*, they relied uniquely on rainfall variability.

2.2 Bacterial inoculation

To obtain rhizobia and *Erwinia* inocula, three bacterial strains were sourced from the laboratory of legumes and sustainable Agrosystems (L2AD) in the Centre of Biotechnology of Borj Cedria (CBBC, Tunisia). The native strains 25PS6 of *Rhizobium laguerreae* sv. *viciae* (Ilahi et al. 2021) was used in Rh treatment. In addition, strains 12PS6 and 13PS9 of *Erwinia* (Ilahi et al. 2024) were used in PG Rh treatments. Every single strain was grown separately to the late exponential phase in yeast-extract-mannitol broth (Vincent, 1970).

2.3 Agronomic parameters and nitrate content in pea leaves

At maturity, *i.e.*, five months after the beginning of the experiment, three plants for each sub-plots were sampled for agronomic parameters evaluation, for a total of nine plants for each treatment. Analyzed agronomic parameters were shoot length (cm), shoot dry weight (g), plant nitrate content (ppm), pod number (n), pod weight (g), root dry weight (g) and root nodule number (n) of both varieties. For dry weight evaluation, the plants harvested at maturity were weighed, and then dissected into the three analyzed components, including shoots, pods and roots for individual

weighing. Pod weight was evaluated as the average weight of 100 pods. After drying the plant material in an oven at 60°C for three days, the dry weight was recorded. Concerning nitrate content analysis, the middle part of each leaf was grounded using a ceramic mortar and the obtained liquid matrix was used to assess nitrate quantification using the "nitrate check" device Horiba LAQUAtwin NO3-11 Compact Nitrate Ion Meters (AllCaT Instruments, BP 32025, F13845, Vitrolles Cedex France). At root level, nodule number was assessed by count.

2.4 Soil sampling

Soils samples were collected *via* drilling at 10-20 cm depth in March 2021 and 2022 and were used for assessment of physicochemical characteristics, as well as for metabarcoding analysis of AM fungal and bacterial communities. For soil physicochemical characteristic evaluation, 1 kg of soil was collected from three different points at the experimental site and average data values were calculated. For metabarcoding analysis, four/five soil samples were sampled in each subplot and merged to obtain one pooled sample for each treatment of approximately 250 g. All samples were then stored at -80°C.

Analyzed physicochemical soil characteristics included texture (Clay, %, Loam, % and Sand, %), pH, exchangeable Na, Mg, Ca and K (mg/g, mg/L, mg/g and mg/L, respectively), assimilable P (µg/g), exchange capacity (EC, mS/cm), soil organic matter content (SOM, %) and total nitrogen (N, %). Analyses were conducted at the Specialized Unit for Research Support and Technological Transfer at the Biotechnology Center of Borj-Cédria as described by Gritli et al., 2023.

2.5 Statistical analysis

Statistical analysis was conducted with R software v. 4.3.2 on the different agronomic parameters *i.e.,* shoot length part (cm), shoot dry weight (g), plant nitrate content (ppm), pod number (n) and weight (g), root dry weight (g) and root nodule number (n). Three-way ANOVA has been performed to assess the effects of factors “year”, “treatment”, and “variety”, as well as their interaction. Additionally, for each variety, data have been analyzed considering the two years of production (2021 and 2022) separately with two-way ANOVA, considering as factors the “treatment”, the “variety” and their interaction. Post-hoc analysis was performed through Tukey HSD Test with significance level set to $p \leq 0.05$. Heatmaps were produced with R package “pheatmap” to investigate the main AM fungal genera and, separately, the main bacterial genera associated to the different treatments in both years. Moreover, a correlation analysis between the presence of AMF and the concurrent presence of bacteria in the different treatments across both

years has been performed using Pearson method, with thresholds set at $p \leq 0.05$ and *R-square* at 0.75.

2.6 Metabarcoding

Total DNA extraction was performed on 18 samples, corresponding to three replicates for each treatment for each year, by using the DNeasy PowerSoil Pro Kit. The 16S rRNA gene V4 region was amplified using the primers P5_8N_515F and P5_7N_515F along with P7_2N_806R and P7_1N_806R (Caporaso et al., 2011; Moll et al., 2018). For the fungal ITS2 regions, amplification followed the protocol outlined by Prada-Salcedo et al. (2021), utilizing primers P5-5/6N-ITS4 (N{5,6}TCCTCCGCTTATTGATATGC) and P7-3/4N-fITS7 (N{3,4}GTGARTCATCGAATCTTTG). The AMF SSU regions were amplified using a nested PCR approach (Wahdan et al., 2021) with primers GLOMERWT0 (CGAGDWTCATTCAAATTTCTGCCC) and GLOMER1536 (AATARTTGCAATGCTCTATCCCCA), followed by NS31 (TTGGAGGGCAAGTCTGGTGCC) and AML2 (GAACCCAAACACTTTGGTTTCC). Each sample and target region underwent PCR amplification in triplicate (using 40 ng DNA template per PCR reaction) with Kapa HiFi polymerase (KapaBiosystems, Boston, USA). The PCR-triplicates amplicons were pooled and purified with AmpPure XP Beads, then indexed with an additional PCR using Illumina Nextera XT v2 index primers, followed by another purification with AmpPure XP Beads. The concentration of indexed and purified PCR products was measured with a NanoDrop ND-8000 spectrophotometer. DNA from fungal and prokaryotic amplicons was pooled equimolarly. Exact concentrations of the final pools were determined using a Qubit dsDNA-HS Assay, and fragment length and quality were further verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The combined prokaryotic and fungal amplicon pools were prepared for Illumina MiSeq paired-end sequencing. Sample libraries and the control library were diluted and denatured according to the MiSeq Illumina kit instructions and loaded onto an Illumina MiSeq flow cell for paired-end sequencing. Raw reads were analyzed through QIIME 2 pipeline (Bolyen et al., 2019). Sequences trimming was performed with cutadapt v3.4. This step was followed by denoising through dada2 v2021.8.0 and the final assembling into Amplicon Sequence Variants (ASVs). The Maarjam (Opik et al., 2010) and Silva v. 138-99 (Quast et al., 2013) databases for AMF and bacteria, respectively, were used to obtain the taxonomic classification of each ASV. In both cases the output was elaborated to obtain a relative abundance (%) of each ASV in the total amounts of the entire sample. Raw reads from metabarcoding of soil samples were submitted to NCBI SRA database under BioProject ID PRJNA1141918. Two ASV tables were produced for the years 2021 and 2022 for

AMF and bacteria, respectively (Tables S1-2). These ASV tables were used as input for Microbiome Analyst (Dhariwal et al., 2017; Chong et al., 2020) for taxon abundance visualization and diversity calculation. Diversity within samples *i.e.,* alpha diversity was calculated with Chao1 index, while diversity among samples *i.e.,* beta diversity was calculated with Bray-Curtis index. The analysis was performed at Feature-level in both cases and beta diversity was reported in a two-dimensional principal coordinates analysis. Taxon abundance was visualized for each year through Stacked Bar plot at family and order level for AMF and bacteria, respectively. Core analysis was performed to investigate the main taxa composition of each year and of each variety at order level.

3. Results

3.1 Agronomic parameters

All the analyzed agronomic parameters including shoot length, shoot dry weight, nitrate content, pod number, pod weight, root dry weight and root nodule number were significantly affected by the factor "year" (Table S3). Overall, the year 2021 was characterized by a higher plant performance and production compared to year 2022 (Table S3). In both years significant differences between varieties and among treatments were observed (Figg. 1-3). Specifically, all considered agronomic parameters were significantly higher in DP variety plants compared to MK variety and a significant reduction was highlighted comparing CTRL to Rh and to PG Rh treatment in both pea cultivars. The PG Rh treatment had a significant effect on all the agronomic parameters (Figg. 1-3). DP plants subjected to PG Rh showed the highest values compared to all the other treatments (Figg. 1-3).

3.2 Soil chemical-physical properties

The soil was classified as loamy soil, with a pH close to neutrality (7.43). Assimilable P (89.56 µg/g) was comparable to that reported in Hachana et al. (2021). The SOM % was slightly high (8.37 %). On the other hand, the total N % was relatively low (0.22%). The exchangeable Na level of 22.06 mg/g was relatively high, as also a EC of 3.23 mS/cm suggested moderate salinity condition. Detailed information are reported in Table S4.

3.3 Soil AM fungal community

The total number of AM fungal reads obtained from Mateur soils was 599,119, of which 271,518 were identified in 2021 and 327,601 in 2022. A total of 59 taxa at VT level were identified (Table S1). The core of AM fungal community of 2021 was represented by orders Glomerales, Paraglomerales, Diversisporales and Archaeosporales. Despite the presence of a common core, the AM fungal community was characterized by large taxon diversity and abundance among

treatments. Differences in taxon abundance between the two varieties (DP and MK) were evaluated by estimation of alpha diversity, that resulted significantly different (*p*-value 0.017, Fig. 4b). In particular, Paraglomerales order was more abundant in DP, while Archaeosporales in MK. Moreover, differences in beta diversity were highlighted also considering the factors "variety" and "inoculation" (*p*-value 0.001 and *p*-value 0.003, respectively, Fig. S2a-b). At family level (Fig. 4a), DP variety was associated to a higher abundance of Diversisporaceae and Paraglomeraceae, while MK to Claroidoglomeraceae, Acaulosporaceae and Archaeosporaceae. In particular, Claroidoglomeraceae family was more abundant in Rh treatment, while Acaulosporaceae was more abundant in CTRL and Archaeosporaceae in PG Rh ones. Correlation analysis highlighted the presence of different VTs associated to each treatment (Fig. 5a). In particular, *Claroidoglomus* sp. was associated to DP CTRL and MK PG Rh, *Diversispora* sp. to DP PG Rh and MK CTRL, while the highest abundance of *Archaeospora* sp. was recorded under MK Rh.

The core of the AM fungal community in 2022 was represented by orders Paraglomerales, Diversisporales and Glomerales. As for 2021, a significant difference was found for alpha diversity between DP and MK (*p*-value 0.014, Fig. 4c), with MK variety associated to a higher taxon abundance compared to DP. Beta diversity highlighted a significant difference between the two varieties (*p*-value 0.001, Fig. S2c), but not considering the "inoculation" factor (Fig. S2d). Specifically, MK variety showed a higher presence of Archaeosporales compared to DP one variety. Families Claroidoglomeraceae, Archaeosporaceae and Acaulosporaceae were more abundant in MK variety, while in DP a higher abundance of Diversisporaceae and Paraglomeraceae was recorded (Fig. 4a). Abundance analysis among AM fungal VTs and the different treatments highlighted that *Paraglomus* sp. was associated to DP CTRL, DP Rh and MK PG Rh, *Diversispora* sp. to all DP samples and MK CTRL, and *Archaeospora* sp. to MK PG Rh (Fig. 5b).

3.4 Soil bacterial community

The core microbiome of 2021 was characterized by orders Solirubrobacterales, Bacillales, Nitrososphaerales, Rhizobiales and Burkholderiales. Bacterial communities of 2021 did not show statistical differences in alpha and beta diversity, despite an increasing trend in taxon abundance was found when comparing MK to DP variety (Fig. 6b). Moreover, taxon abundance increased from CTRL to Rh treatment, with PG Rh showing intermediate values, except for MK Rh that showed the lowest taxa abundance. No differences were highlighted considering the beta diversity (Fig. S3a). Variety core microbiome showed some differences in taxon associations. Specifically, soil of variety DP was characterized by a high presence of Gaiellales, while MK one by the presence of Rubrobacterales and Frankiales. Taxon abundance at order level (Fig. 6a) showed that

most of the taxa had a similar trend in both varieties and among the different treatments. Despite that, some orders were more abundant in soil of DP compared to MK one and vice versa. In particular, soil of DP showed a higher abundance of Gaiellales, Burkholderiales, Thermomicrobiales, Tistrellales, Propionibacterales, Sphingomondales and Streptomycetales. On the other hand, soil of MK showed a higher abundance of Rubrobacterales, Rhizobiales, Frankiales and Solirubrobacterales. Correlation analysis between the different treatments and the associated bacterial genera highlighted the presence of some representative taxa (Fig. 7a). *Bacillus* and *Solirubrobacter* represented the main taxa in all DP soil samples and in MK CTRL, while *Domibacillus* was uniquely associated with MK Rh one. Moreover, *Rubrobacter* was associated to all MK soil samples and DP Rh and *Solirubrobacter* to DP CTRL. The highest abundance of *Bacillus* was detected in DP PG Rh and MK CTRL samples, while the highest abundance of *Rubrobacter* in MK PG Rh ones.

The core microbiome of bacterial community of 2022 was represented by orders Gaiellales, Solirubrobacterales, Rubrobacterales, Bacillales and Tistrellales. Samples showed variation alpha diversity considering the "inoculation" factor (*p*-value 0.024) and the interaction between "inoculation" and "variety" factors (*p*-value 0.03, Fig. 6b), while no differences were highlighted in beta diversity (Fig. S3b). Not-treated samples (CTRL) showed an overall lower associated-taxa diversity compared to the others. The highest taxon diversity was recorded in MK soil under PG Rh treatment. It is interesting to notice that, in DP soil, taxon diversity increased progressively from CTRL to Rh treatment, with PG Rh with intermediate values. The core microbiome of DP soil samples showed specific orders associated to DP variety *i.e.*, Propionibacterales, Micrococcales and Rhizobiales. On the other hand, the core microbiome of MK soil samples showed as uniquely associated taxa the orders Frankiales, Microtrichales, Nitrososphaerales and Vicinamicrobiales. Soil samples of MK variety showed a higher diversity among and inside treatments. Taxon abundance visualization (Fig. 6a) revealed that, in DP variety soil, orders Gaiellales, Rubrobacterales, Micrococcales, Gemmatimonadales, Propionibacterales and Tistrellales were more abundant than in MK one, in which, on the contrary, orders Vicinamibacterales, Frankiales, Microtrichales, Thermomicrobiales, Nitrososphaerales and Azospirillales were more abundant. Abundance analysis performed between the different treatments and the associated bacterial genera (Fig. 7b) showed that *Bacillus* and *Rubrobacter* were the most abundant taxa in the different treatments, in particular *Bacillus* higher abundance was detected in DP PG Rh and *Rubrobacter* in DP CTRL, DP Rh and MK PG Rh. Additionally, *Blastococcus* was associated with MK Rh samples.

3.5 Comparison between the two years and correlation between AM fungal and bacterial taxa

The AM fungal core microbiome was conserved between 2021 and 2022, with the exception of Archaeosporales that were absent in 2022 core. Comparison of AM fungal abundance performed between the two years highlighted that all the most abundant families were conserved between the two years (Fig. 4). In particular, DP variety soil showed a higher abundance of Diversisporaceae and Paraglomeraceae, while in MK soil, families Claroideoglomeraceae, Acaulosporaceae and Archaeosporaceae were the most abundant ones. Abundance analysis highlighted that *Claroideoglomus* genus was always associated to MK variety soil, despite its abundance varied among the different treatments of this variety (Fig. 5).

Bacterial core microbiome showed that Solirubrobacterales and Bacillales were conserved between the two years, while the abundance of other taxa varied. In particular, in both years DP variety soil showed a higher abundance of Gaiellales, Propionibacterales and Tistrellales (Fig. 6a). On the other hand, MK variety soil was characterized by the associated presence of Frankiales (Fig. 6b). Abundance analysis revealed no conserved genera associated to the different treatments in the two years (Fig. 7). Overall, most of the associated taxa did not show variation in relation to the variety, the inoculation and the year factors.

Correlation analysis performed between AM fungal and bacterial community showed different correlated taxa in the two varieties soils (Fig. 8). Specifically, DP variety soil was characterized by a higher number of correlated taxa with respect to MK one. In DP variety soil, *Ambispora*, *Otospora* and *Racocetra* were correlated with *Microbacterium* and *Skermanella* in PG Rh treatment. Moreover, in PG Rh treatment and in Rh, *Archaeospora* was correlated with *Roseisolibacter*. *Entrophospora* was correlated with *Bryobacter*, *Domibacillus*, *Dongia*, *Massilia*, *Noviherbaspirillum* and *Tumebacillus* in CTRL. *Scutellospora* was correlated with *Gemmatimonas* in PG Rh and Rh treatments. In all treatments of DP variety *Glomus* was correlated with *Microlunatus*, while *Paraglomus* was correlated with *Rubrobacter* (Fig. 8a). Similarly to DP, in MK variety soil the correlations highlighted between *Glomus* and bacterial taxa were detected only in CTRL treatment. In particular, *Glomus* was correlated with *Gemmatimonas*, *Microbacterium* and *Noviherbaspirillum*. In all treatments, *Acaulospora* was correlated with *Bacillus* and *Scutellospora* with *Blastococcus*. Additionally, *Scutellospora* was also correlated with *Sphingomonas* in PG Rh and Rh treatments (Fig. 8b).

4. Discussion

4.1 Bacterial inocula positively affected agronomic traits in both pea varieties

The two-year trial here conducted in open field conditions allowed to explore the response of two different pea varieties after rhizobia and PGPB inoculation, as well as the interaction between pea plants and soil microbiota, focusing on natural AM fungal and bacterial communities. Based on agronomic parameters such as shoot length, shoot dry weight, number of pods, and pod weight, the performances of pea plants in 2021 were generally higher to that observed in 2022. A similar difference was recorded also considering the variety effect, with DP plants that were characterized by higher agronomic parameters compared to MK one. However, for both varieties, the Rh and PG Rh inoculation had a positive impact on all the agronomic parameters, especially on shoot dry weight, pod number and weight. Notably, results on roots showed that PG Rh inoculation was associated to a higher number of nodules, as well as to higher root dry weight, compared to the other inoculation conditions. In Tunisia, these results were similar to those reported by Hachana et al. (2021). The authors found that different pea varieties exhibited varying levels of adaptability to soil variations, with the long cycle variety showing the highest nodulation and adaptability to soil conditions (Hachana et al., 2021). A study of Abebaw (2024) performed in Ethiopia have shown that rhizobia inoculation significantly enhanced various agronomic parameters in *P. sativum*, including pod number, seed weight, and total seed yield. Another research highlights the potential of PGPB, specifically a *Pseudomonas* strain with PGP traits to enhance pea growth under water deficit conditions, involving physiological and metabolic shifts that increase ABA content in pea shoots (Schillaci et al., 2024). Concerning leaf nitrate content, it has been previously demonstrated that PGPB inoculation is generally associated a higher root nitrate absorption (Aquino et al., 2021). Our results showed that leaf nitrate content was higher in inoculated samples. This results can be explained by the fact that during the flowering stage of plant growth, plants require more nitrogen to support the development of flowers (Yun et al., 2023) and different studies demonstrate that PGPB are able to boost root N-absorption (Di Benedetto et al, 2017). As the plant transitions from the flowering stage to the pod formation stage, the nitrate concentration typically decreases. This reduction occurs because the plant shifts its focus from vegetative growth to reproductive development. During the pod stage, the plant reallocates its resources to the production and maturation of pods and seeds. As a result, the demand for nitrogen decreases, leading to lower nitrate levels in the plant tissues (Rahmat et al, 2023).

Overall, considering all the analyzed agronomic parameters, PG Rh treatment, in which the *Erwinia* strains have been used, resulted in improved plant performance and higher agronomic parameters compared to both control and Rh ones, suggesting a beneficial role in promoting pea plants growth. *Erwinia* belongs to Enterobacterales (Adeolu et al., 2016), that includes several PGPB

(Ramakrishnan et al., 2023) and the used strains have selected because of their PGP activity (Ilahi et al., 2024).

4.2 Soil AM fungal and bacterial alpha diversity increased in presence of the bacterial inoculation

In both years, in PG Rh inoculated parcels, higher AM fungal and bacterial diversity was detected with respect to control ones, suggesting a role of the inocula in improving microbiome diversity and abundance in soil. Most of the AMF identified in this study have been previously reported in arid and semi-arid environments, and include *Glomus* sp., *Scutellospora* sp., *Ambiospora* sp., *Diversispora* sp. and *Paraglomus* sp., with *Glomus* sp. as the most distributed genus across the different environments (Torrecillas et al., 2012; M'saouar et al., 2019; Gritli et al., 2020; Guo et al., 2021; Ibou and Fatou, 2021; Ashwin et al., 2023). Notably, in our study the order Glomerales represented the AMF core identified for both varieties, as previously documented in Tunisian soils (Gritli et al., 2020). Additionally, variety-dependent AM fungal associations were identified and specifically, in soil of MK variety a higher abundance of Archaeosporales, Claroidoglomerales and Acaulosporales was observed, while in DP variety the presence of Diversisporales and Paraglomerales was recorded. *Diversispora* and *Claroideoglomus* genera have been previously reported to be abundant in field of pea plants, and *Diversispora* has been suggested as a key group of AMF recruited by pea (Lee et al., 2023), as also observed for DP variety in our experiment. Different studies highlighted that AM fungal diversity can be also associated to P levels in the soil (Chu et al., 2013; Baltruschat et al., 2019; Xiao et al., 2019). A recent meta-analysis on global distribution of AMF has been suggested that limited P availability leads to increased AM fungal diversity (Ma et al., 2023). In our study, the soil showed an average level of P (89.56 μg/g), and this could explain the detected diversity in AM fungal communities, as previously observed (Chu et al., 2013; Xiao et al., 2019). Thus, considering the increase in AM fungal diversity triggered by Rh and PG Rh inoculation, it cannot be excluded that the identified improvement in pea agronomic parameters may be due to both bacterial inocula and specific AM fungal associations established by each variety.

Concerning the bacterial communities, in soil of MK variety was found with a higher presence of the order Frankiales, while in DP one a higher abundance of Gaiellales and Propionibacterales was recorded. It is noteworthy that Frankiales, Gaiellales, and Propionibacterales are members of the Actinobacteria phylum (Dworkin et al., 2006; Albuquerque and da Costa 2014; Narayanasamy et al., 2020), a well-known group of bacteria that can be found in the mycorrhizosphere (Agnolucci et al., 2019; Ujvári et al., 2021) and that can act as PGPB (Caddell et al., 2020). Actinobacteria is

reported as a group of bacteria that can improve plant fitness through several mechanisms, including inorganic soil phosphorus solubilization, nitrogen-fixing ability, as well as production of ACC-deaminase, siderophores, phytohormones and enzymes that may regulate plant growth (Mitra et al., 2023). Among Actinobacteria, the order Frankiales harbors taxa known to be able to establish symbiotic relationships with plant roots and also to live in soils as saprotroph (Mantelin and Touraine, 2004). They are considered the only Actinobacteria taxon that have N_2 fixing ability (Bouizgarne and Ait Ben Aouamar, 2014) and have proved to have drought-resistant characteristics (Gupta et al., 2020). Interestingly, these bacteria are able to establish symbiotic relationships with a group of plants named actinorhizal plants (Benson and Dawson, 2007), which comprise non-leguminous plants belonging to eight angiosperm families and 24 genera (Narayanasamy et al., 2020). Considering that pea is a leguminous crop, the presence of Frankiales in soil of MK variety may, therefore, be explained by the presence of specific AMF rather than on the relationship with pea plants.

4.3 Different putative PGPB were associated with specific AM fungal groups

In our study, different correlations among AMF and bacteria were highlighted in both soils of DP and MK varieties. Specifically, in soil of DP variety *Microbacterium* and *Skermanella* were correlated with the AMF *Otospora* and *Racocetra*, while *Roseisolibacter* was found to be correlated with *Archaeospora*. The genus *Microbacterium* included strains considered as PGPB able to enhance shoot fresh weight in leguminous plants such as *Trifolium pratense* (Martínez-Hidalgo and Hirsch, 2017). Its PGP activity relies on different mechanisms, including ACC deaminase and IAA production, plant ethylene reduction and the production of antifungal metabolites able to contrast plant pathogens (Chieb and Gachomo, 2023; Tsavkelova et al., 2024). This genus has also been observed as part of the endophytic community in pea seeds, along with *Bacillus*, *Sphingomonas*, and *Erwinia* (Chartrel et al., 2021). *Skermanella* genus includes mycorrhizal helper diazotrophic bacteria able to perform biological N-fixation (Nasuelli et al., 2023). *Skermanella* can act as a mycorrhizal rhizosphere indicator and can perform as PGPB *via* N-fixation and production of alkaline phosphatase (Chen et al., 2023). This genus has previously been reported as correlated with the presence of *Gaiella,* and this co-occurrence has been suggested as a driver of the improvement of the N-uptake in wheat plants grown in N-rich conditions (Chen et al., 2023). Notably, in our study the order Gaiellales, which includes *Gaiella* genus, was more abundant in soil of DP variety for which the correlation among *Skermanella* and the different AM fungal genera has been identified. *Roseisolibacter* belongs to Gemmatimonadaceae family (Zhang et al., 2003; Pascual et

al., 2018) that includes P-solubilizers and bacteria able to induce plant hormone production (You et al., 2024).

On the other hand, in soil of MK variety, the AMF *Scutellospora* and *Glomus* were found to be correlated with *Sphingomonas* and *Gemmatimonas,* respectively. *Sphingomonas* includes diazotrophic bacteria (Nasuelli et al., 2023). This genus has been reported to be able to produce IAA (Chieb and Gachomo, 2023) and to improve shoot length and dry weight in soybean (Asaf et al., 2017). Moreover, its abundance seems to increase in *Lotus japonicus* rhizosphere in presence of AMF including *Glomus mosseae*, *Rhizophagus intraradices*, and *G. versiforme* (Xu et al., 2023). The genus *Gemmatimonas* has been previously observed as enriched in *L. japonicus* rhizosphere under AM fungal inoculation (Xu et al., 2023).

5. Conclusions

In conclusion, our study demonstrated the positive effect of inoculation with rhizobia and the *Erwinia* strains on two pea cultivars in field conditions. Inoculation with these bacteria positively impacted all agronomic parameters, and DP variety showed higher performance compared to MK. High bacterial and AM fungal diversity in soil of PG Rh inoculated conditions was observed, suggesting that this bacterial consortium enhances the soil microbial diversity and abundance. The analysis of the soil bacterial core microbiome revealed distinct associations for each pea variety and, particularly, some taxa belonging to Actinobacteria reported as PGPB were identified. This suggested that the specific bacterial communities, associated with each variety, may play a crucial role in promoting plant growth and productivity. Notably, it has been reported that AM fungal mycorrhizosphere hosts different bacterial taxa, including bacterial strains recognized for their PGP traits (Agnolucci et al., 2019). Correlations between several bacterial and AMF taxa, *e.g., Microbacterium* and *Skermanella* and the AMF taxa *Otospora* and *Racocetra* in the DP cultivar, as well as *Sphingomonas* and *Scutellospora* in MK cultivar, suggested a relevant role of the microbial communities of the mycorrhizosphere in improving both soil health and pea plant performance. Overall, our study highlights the potential of tailored inoculation approaches to improve pea plant growth and productivity by exploiting the synergistic effects of soil microbial interactions in the frame of sustainable agriculture practices.

CRediT authorship contribution statement

Conceptualization: BM; Methodology: RB, FS, TR, SD, MMEI, LO, MNA, BM; Investigation: HI, AC, RB, BM, FS; Formal analysis: HI, AC, FS, TR; Writing—Original Draft: HI, AC, FS; Writing—Review & Editing: AC, FS, RB, TR, BM; Supervision: RB, BM.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Reads from metabarcoding of soil samples are available at NCBI SRA database under BioProject ID PRJNA1141918. All other data are included in the manuscript and its Supplementary materials.

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Figure captions

Fig. 1. Shoot agronomic parameters of pea plants. a-b) Shoot length (cm), c-d) shoot dry weight (g) and e-f) leaf nitrate content (ppm) of DP and MK varieties in 2021 and 2022 subjected to the three different inoculation conditions (n=9). ANOVA p-values are included (cv = cultivar, inoculation = inoculation condition, cv x inoculation = interaction). Ns, *, **, ***: not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Different letters showed significant differences according to Tukey HSD test ($p \leq 0.05$), considering the associated significant source of variance in year 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not -inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Fig. 2. Pea pod production. a-b) Pod number (n) and c-d) pod weight (g) of DP and MK varieties in 2021 and 2022 subjected to the three different inoculation conditions (n=9). ANOVA p-values are included (cv = cultivar, inoculation = inoculation condition, cv x inoculation = interaction). Ns, *, **, ***: not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Different letters showed significant differences according to Tukey HSD test ($p \leq 0.05$), considering the associated significant source of variance in year 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Fig. 3. Root agronomic parameters of pea plants. a-b) Root dry weight (g) and c-d) root nodules number (n) of DP and MK varieties in 2021 and 2022 subjected to the three different inoculation conditions (n=9). ANOVA p-values are included (cv = cultivar, inoculation = inoculation condition, cv x inoculation = interaction). Ns, *, **, ***: not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Different letters showed significant differences according to Tukey HSD test ($p \leq 0.05$), considering the associated significant source of variance in year 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not -inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Fig. 4. AM fungal diversity and abundance in soil of DP and MK varieties subjected to the three inoculation conditions. a) AM families abundance in the two varieties in 2021 and 2022, b)

alpha diversity of AM fungal community in 2021 and c) alpha diversity of AM fungal community in 2022. Alpha diversity analysis (Chao1 index) was performed considering the interaction between the "variety" and the "inoculation" factors. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Fig. 5. Heatmap of the most abundant AM fungal taxa at genus level. a) AM fungal taxa abundance in soil of DP variety subjected to the three inoculation conditions in 2021 and 2022, and b) AM fungal taxa abundance in soil of MK variety subjected to the three inoculation conditions in 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Fig. 6. Bacterial diversity and abundance in soil of DP and MK varieties subjected to the three inoculation conditions. a) Bacterial orders abundance in the two varieties in 2021 and 2022, b) alpha diversity of bacterial community in 2021 and c) alpha diversity of bacterial community in 2022. Alpha diversity (Chao1 index) analysis was performed considering the interaction between the "variety" and the "inoculation" factors. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Fig. 7. Heatmap of the most abundant bacterial taxa at genus level. a) Bacterial genera abundance in soil of DP variety subjected to the three inoculation conditions in 2021 and 2022, and b) bacterial genera abundance in soil of MK variety subjected to the three inoculation conditions in 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Fig. 8. Correlation analysis between AM fungal and bacterial communities in soils of the two varieties. a) Correlation analysis (Pearson correlation) between AM fungal and bacterial communities in soil of DP variety and b) correlation analysis (Pearson correlation) between AM fungal and bacterial communities in soil of MK variety. DP: Douce de Provence, MK: Merveille de Kelvedon. R squares and p-values are reported.

Supplementary materials

Supplementary tables

Table S1. Amplicon sequence variant (ASV) table of AMF in Mateur soil.

Table S2. Amplicon sequence variant (ASV) table of bacteria in Mateur soil.

Table S3. Agronomic parameters. Shoot length (cm), shoot dry weight (g), leaf nitrate content (ppm), pod number (n), pod weight (g), root dry weight (g) and root nodules number (n). All results are reported as mean ± standard deviation. Ns, *, **, ***: not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Different letters showed significant differences according to Tukey HSD test ($p \leq 0.05$), considering the associated significant source of variance in year 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh : inoculated with *Rhizobium* strain 25ps6, PG Rh : inoculated with *Rhizobium* 25ps6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Table S4. Soil chemical-physical properties.

Supplementary figures

Fig. S1. Experimental design. The first plot was used for Douce de Provence variety and the second one for Merveille de Kelvedon one. CTRL: not-inoculated condition, Rh: inoculated with Rhizobium strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains

Fig. S2. AM fungal abundance in Mateur soil. a) Beta diversity performed considering the "variety" factor in year 2021, b) beta diversity performed considering the "inoculation" factor in year 2021, c) beta diversity performed considering the "variety" factor in year 2022 and d) beta diversity performed considering the "inoculation" factor in year 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh : inoculated with *Rhizobium* strain 25ps6, PG Rh : inoculated with *Rhizobium* 25ps6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

Fig. S3. Bacterial abundance in Mateur soil. a) Beta diversity performed considering the "variety" factor in year 2021 and b) beta diversity performed considering the "variety" factor in year 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh : inoculated with *Rhizobium* strain 25ps6, PG Rh : inoculated with *Rhizobium* 25ps6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

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