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Characterization of endophytic bacteria isolated from root nodules of lentil in intercropping with durum wheat

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ABSTRACT

Legumes improve soil fertility by interacting symbiotically with nitrogen-fixing rhizobia allocated in root nodules. Some bacterial endophytes can coexist with rhizobia in nodules and might help legumes by enhancing stress tolerance, producing hormones stimulating plant growth, and increasing plant nutrient intake. Twenty-six bacterial endophytes from *Lens culinaris* root nodules cultivated in intercropping with *Triticum durum* were identified and characterized molecularly and biochemically. Potential plant growth-promoting strains have been selected according to the indole acetic acid and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production, and for their inorganic phosphate solubilization ability. The presence of genes associated to ACC deaminase and nitrogenase was evaluated. Six selected strains were grown with varying NaCl and polyethylene glycol concentrations to test their salt and osmotic stress tolerance. *Priestia megaterium* 11NL3 and *Priestia aryabhattai* 19NL1, resulted to be tolerant to salinity and osmotic stress, were tested on four genotypes of *T. durum* seeds in different stress conditions. The effect of strain inoculation on seed germination, vigor, and root-to-shoot ratio varied depending on the type of stress and on the durum wheat genotypes. For future research, it will be necessary to test the selected bacterial strains at different plant phenological stages and to clarify the mechanisms involved in the different outcomes of plant-microbe interactions.

1. Introduction

Since the origins of agriculture, pulse crops have contributed to maintain soil fertility due to their symbiotic association with nitrogen-fixing rhizobia in root nodules (Zahran, 2001). Even if rhizobia are the primary inhabitants, some bacterial endophytes can coexist with them in nodules and may have plant growth-promoting (PGP) traits (Muresu et al., 2019). Although several abiotic stress factors may impair the ability of legumes to successfully establish a symbiosis with rhizobia, endophytic bacteria may improve plant nutrition and growth by providing tolerance to biotic and abiotic stresses (Zamioudis and Pieterse, 2012). In particular, stress conditions induce ethylene production in plants, negatively influencing their growth and productivity (Dubois et al., 2018; Wei et al., 2018), and reducing nodule formation (Lee and Larue, 1992; Gresshoff et al., 2009; Ferguson and Mathesius, 2014). However, the presence of plant growth-promoting rhizobacteria (PGPR) able to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase may mitigate ethylene adverse impacts (Singh et al., 2015). This enzyme in fact sequesters and converts the ethylene precursor ACC into ammonia. As a result, the accumulation of high concentrations of ethylene in plant tissues is avoided, resulting in an enhanced resistance to stress and facilitating the symbiosis with rhizobia (Shaharoona et al., 2011). In addition, endophytic PGPR may act as biofertilizers, increasing in different ways plant nutrient uptake. The employment of microorganisms that can increase nutrient availability is an intriguing approach to lessen the harmful impacts of chemical fertilizers on the environment, such as eutrophication of water sources (Liu et al., 2021). Furthermore, the world's mineral P resources are finite and are predicted to be exhausted within 100 years (Gilbert, 2009; Herrera-Estrella and López-Arredondo, 2016). Phosphate-solubilizing bacteria are used as biofertilizers as they provide an essential macronutrient that is involved in all metabolic pathways, including photosynthesis, to the plant (Wang et al., 2021). In particular, in legume plants, P is also related to the nitrogen fixation process (Kouas et al., 2005). The production of plant hormones is another important mechanism implemented by certain bacteria in their activity of PGP. For instance, indole acetic acid (IAA) is involved in plant growth, promoting cell division and root elongation (Majda and Robert, 2018; Li et al., 2022).

For all these reasons, PGPR are being studied extensively, and their

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Abbreviations

aminocyclopropane-1-carboxylic acid (ACC)
plant growth-promoting (PGP)
plant growth-promoting rhizobacteria (PGPR)
indole acetic acid (IAA)
Luria-Bertani Agar (LBA)
colony forming unit (CFU)
Phosphorus (P)
Phosphate Solubilization Index (PSI)
phosphate solubilization efficiency (PE)
Luria-Bertani broth (LB)
Dworkin & Foster medium (DF)
potato dextrose agar (PDA)
nutrient broth added with glucose (NBG)
polyethylene glycol (PEG)
germination index (GI)
seed vigor index (SVI)
root-to-shoot ratio (R/S)
not determined (nd).

use in modern agriculture has increased over the last two decades (Calvo et al., 2014; Basu et al., 2021). Indeed, there is growing awareness that global climate change is having a negative influence on agriculture and drought and salinity are two major factors that are nowadays challenging crop production, especially in arid and semi-arid areas (Sabagh et al., 2020). The PGPR isolated from stressful environments have improved endurance to stress conditions compared to those isolated from optimal habitats (Okoro et al., 2009; Upadhyay et al., 2009; Patel et al., 2017). Plant-associated beneficial strains inhabiting arid and harsh ecosystems may help plants survive under abiotic and biotic stress factors, as these microbes have developed complex adaptive traits in co-evolution with their plant hosts, in contrast to those found in frequently cultivated areas (Fierer, 2017).

The aims of this study were to isolate and characterize, both molecularly and biochemically, root nodule-inhabiting bacterial endophytes associated with *Lens culinaris* grown in intercropping with *Triticum durum* and to select promising plant growth-promoting strains based on molecular and biochemical *in vitro* characterization.

2. Materials and methods

2.1. Isolation of bacterial endophytes from lentil nodules

Lens culinaris (Eston genotype) plants were cultivated in an open field trial in intercropping with durum wheat at the Azienda Pantanello, located in Metaponto, Southern Italy (40°23′031.4″ N, 16°47′010.9″ E). The experimental field had an electrical conductivity of less than 1 dS m^{-1}. The plants were cultivated in rainfed agriculture. Lentil root nodules were collected from 12 plants in April 2022. The root nodules were separated from the roots with a sterile scalpel, washed and surface sterilized following the protocol of Efstathiadou et al. (2020). Briefly, root nodules were washed in sterile distilled water to remove adhering soil. Subsequently, each root nodule was placed in a 2 mL microcentrifuge tube with 1 mL of Ethanol 70 % (v/v) for 60 s and immediately washed in 1 mL of sterile distilled water and the wash step was repeated five times. To check the surface sterilization success, 100 μl of water from the last wash were distributed onto a Petri dish containing LBA (Luria-Bertani Agar; 90 mm Ø) and incubated for three days at 28 °C in the dark. The surface-sterilized root nodules were crushed using micro pestles in the microcentrifuge tubes with the remaining 900 μl of water. The resulting suspension was serially diluted 1:10 for seven times in sterile distilled water, 100 μl of each dilution (from 10^{-2} to 10^{-7}) were spread onto LBA Petri dishes and the plates were incubated at 28 °C in the dark for three days. Single colonies of bacteria, which were morphologically different from one another, were re-isolated on new LBA dishes to obtain pure cultures and long-term stored in glycerol 40 % (v/v) at −80 °C. The strains used in this study, here identified by the local collection codes, were also deposited and are publicly available at the Agro-Food Microbial Culture Collection ITEM of the Institute of Sciences of Food Production (Italian National Research Council) (local and ITEM strain codes are reported in Table 1).

2.2. Preparation of bacterial cell suspensions

Bacterial strains were routinely cultivated at 27 °C on LBA in Petri plates (90 mm Ø). Bacterial cells were collected from LBA plates after 48 h incubation at 27 °C and suspended in 1 mL of sterile saline solution (0.85 % NaCl w/v) contained into sterile 2 mL microcentrifuge tubes. The cell suspension was centrifuged (13,000 rpm, 1 min), the supernatant was removed and the cells were suspended in 1 mL of sterile saline solution. The washing was repeated twice to eliminate any trace of nutrients from LBA and secondary metabolites produced by bacterial growth. Finally, pelleted bacterial cells were suspended into sterile water, adjusted to a final concentration of 1×10^8 colony forming units per volume unit (CFU mL^{-1}) and utilized in all experiments.

2.3. Molecular characterization of bacterial isolates

The total DNA was extracted from the isolated bacteria with the commercial kit E.Z.N.A.® Bacterial DNA (OMEGA Bio-Tek) according to manufacturer's instructions. The identification of the isolated bacteria was done through the amplification of the 16S rRNA gene sequence, using the primers 27F-1495R (Frank et al., 2008). The PCR reaction was set with the following steps: 95 °C for 2 min, 94 °C for 30 s, 55 °C for 30 s, 92 °C for 90 s (35 cycles), 72 °C per 7 min. The obtained amplicons were checked on a 1 % agarose gel in 0.5X TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0) stained with GelRed® (Biotium) and compared with the GeneRuler 100 bp DNA ladder (ThermoFisher Scientific, Waltham, MA, USA). The amplicons were purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced at Ludwig Maximilian University Sequencing Service (Martinsried, Germany). The obtained chromatograms were analyzed and edited in the Chromas 2.6.6 software. The sequences were compared with those deposited in the NCBI database with Blastn in the rRNA/ITS databases, excluding uncultured/environmental sample sequences, limiting to sequences from type material. Sequence alignments and phylogenetic analyses were conducted through the software MEGA X (Kumar et al., 2018). Sequences were aligned with the MUSCLE algorithm and a phylogenetic tree was built with the Maximum Likelihood method using 1000 Bootstrap replicates (Felsenstein, 1985; Tamura and Nei, 1993).

2.4. Characterization of plant growth-promotion traits of the selected bacterial isolates

The presence of the gene related to 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (*acdS*) was assessed with a PCR reaction using the primers 1936F (5′-GHGAMGACTGCAAYWSYGGC-3′) and 1939R (5′-GARGCRTCGAYVCCRATCAC-3′) (Blaha et al., 2006) with the Platinum™ II Hot-Start PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA). PCR settings for 1936F-1939R amplification were the following: 3 min at 94 °C; 30 s at 94 °C, 30 s at 58 °C, 90 s at 72 °C (35 cycles); 7 min at 72 °C. The presence of genes related to nitrogenase (*nifH*) in the selected bacterial genome was assessed with the specific primers 107F64 (5′-TGYGAYCCSAARGCNGACTC-3′) and 379R64 (5′-GGCATNGCRAARCCRCCRCA-3′) (Wang et al., 2022). PCR settings for 107F64–379R64 amplification were the following: 3 min at 94 °C; 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C (35 cycles); 7 min at 72 °C. The

Table 1
Identification of bacterial endophytes isolated from lentil root nodules. The local and ITEM Culture Collection strain IDs, the isolation source, the identification based on NCBI Blastn (database rRNA_typestrains/16S_ribosomal_RNA, accessed on 17 March 2023) and the NCBI accession numbers of the partial 16S rRNA sequences are reported for each endophytic strain isolated in this study.

Strain local ID	ITEM strain ID	Isolation source	Identification	NCBI accession
10NL1	–	*Lens culinaris* (Eston genotype) root nodules	*Acinetobacter calcoaceticus*	OR144218
18NL1	19,259	*Lens culinaris* (Eston genotype) root nodules	*Arthrobacter* sp.	OQ686743
11NL4	19,277	*Lens culinaris* (Eston genotype) root nodules	*Bacillus pseudomycoides*	OQ686723
16NL1	19,278	*Lens culinaris* (Eston genotype) root nodules	*Bacillus pseudomycoides*	OQ686729
9NL3	19,262	*Lens culinaris* (Eston genotype) root nodules	*Bacillus pseudomycoides*	OQ686734
11NL1	19,275	*Lens culinaris* (Eston genotype) root nodules	*Bacillus* sp.	OQ686730
18NL4	19,269	*Lens culinaris* (Eston genotype) root nodules	*Microbacterium hydrocarbonoxydans*	OQ686740
13NL2	19,265	*Lens culinaris* (Eston genotype) root nodules	*Niallia* sp.	OQ686724
17NL1	19,256	*Lens culinaris* (Eston genotype) root nodules	*Paenarthrobacter nitroguajacolicus*	OQ686722
9NL2	19,276	*Lens culinaris* (Eston genotype) root nodules	*Paenarthrobacter* sp.	OQ686731
18NL2	19,267	*Lens culinaris* (Eston genotype) root nodules	*Paenarthrobacter* sp.	OQ686738
18NL3	19,268	*Lens culinaris* (Eston genotype) root nodules	*Peribacillus frigoritolerans*	OQ686739
12NL1	19,257	*Lens culinaris* (Eston genotype) root nodules	*Peribacillus frigoritolerans*	OQ686744
20NL2	19,272	*Lens culinaris* (Eston genotype) root nodules	*Peribacillus* sp.	OQ686725
11NL2	19,264	*Lens culinaris* (Eston genotype) root nodules	*Peribacillus* sp.	OQ686736
17NL3	–	*Lens culinaris* (Eston genotype) root nodules	*Plantibacter flavus*	OR144220
14NL1	–	*Lens culinaris* (Eston genotype) root nodules	*Plantibacter flavus*	OR144219
14NL2	19,266	*Lens culinaris* (Eston genotype) root nodules	*Priestia aryabhattai*	OQ686737
19NL1	19,270	*Lens culinaris* (Eston genotype) root nodules	*Priestia aryabhattai*	OQ686741
9NL1	19,261	*Lens culinaris* (Eston genotype) root nodules	*Priestia megaterium*	OQ686733
11NL3	19,263	*Lens culinaris* (Eston genotype) root nodules	*Priestia megaterium*	OQ686735
19NL2	19,260	*Lens culinaris* (Eston genotype) root nodules	*Pseudomonas koreensis*	OQ686732
17NL2	19,273	*Lens culinaris* (Eston genotype) root nodules	*Pseudomonas koreensis*	OQ686726
10NL2	19,274	*Lens culinaris* (Eston genotype) root nodules	*Pseudomonas koreensis*	OQ686728
15NL1	19,258	*Lens culinaris* (Eston genotype) root nodules	*Psychrobacillus* sp.	OQ686727
20NL1	19,271	*Lens culinaris* (Eston genotype) root nodules	*Rossellomorea marisflavi*	OQ686742

obtained amplicons were checked on 1.5 % agarose gel.

2.5. Biochemical characterization of bacterial isolates

The bacterial strains were biochemically characterized by performing tests for inorganic phosphate solubilization, indole acetic acid production and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase residual. The ability of bacteria to solubilize insoluble phosphate was determined by spot inoculating 5 μL of bacterial cell suspension (1×10^8 CFU mL^{-1}) on Pikovskaya agar medium plates (Himedia laboratories, India) containing tri-calcium phosphate ($Ca_3(PO_4)_2$). Three replicates were prepared for each Petri plate. After incubation at 28 °C for 72 h, the occurrence of clarification haloes around bacterial colonies was considered as a positive result for phosphate solubilization activity, and haloes diameters were measured using Fiji software (ImageJ1.50i; Schneider et al., 2012) on the live digital images. The Phosphate Solubilization Index (PSI) was calculated as (colony + halo diameter)/colony diameter (Pande et al., 2017) and the Phosphate solubilization efficiency (PE) was obtained from the ratio between halo and colony diameters (Rokhbakhsh-Zamin et al., 2011).

Indole-3-acetic acid (IAA) production was evaluated by a colorimetric detection test in liquid culture (Karnwal, 2009). Isolates were pre-cultured in Luria-Bertani (LB) broth, and 300 μL of cell suspension (adjusted to 1×10^8 CFU mL^{-1}) of each isolate were used to inoculate 15 mL tubes containing 3 mL of DF salt minimal broth amended with 150 μg mL^{-1} of L-Tryptophan (Merck, Italy). Tubes were incubated at 28 ± 2 °C on an orbital shaker (200 rpm) for 48 h in the dark. A volume of 1 mL of bacterial cell suspensions was centrifuged (4000 rpm, 20 min, 4 °C) and 250 μL of supernatant were mixed with one mL of Salkowski's reagent (1.2 % $FeCl_3$ in 37 % sulphuric acid). After 20 min incubation at room temperature, 96-well polystyrene dishes (Sarstedt, Germany) were used to accommodate the reaction mixtures (two aliquots of 150 μL for each isolate) and the untreated bacterial suspensions (two aliquots of 150 μL for each isolate). Final cell density of bacterial suspensions was determined by measuring the absorbance at 600 nm and the intensity of resulting pink color in the reaction mixtures was quantified by measuring the absorbance at 530 nm in a FluoStar Omega microplate reader (BMG LABTECH, USA). Concentration of IAA was determined by a standard curve prepared from pure IAA solutions (Merck, Italy) in the range 0.5–100 μg mL^{-1}. The amount of bacterial IAA was expressed as μg IAA produced by cell suspensions with 0.5 McFarland scale density (1.5×10^8 CFU mL^{-1}).

To assess ACC utilization by bacterial isolates (Li et al., 2011), aliquots (2 mL) of LB cultures were centrifuged at 8000 g for 5 min, the pellets were washed twice with 1 mL of DF medium (Dworkin and Foster, 1958) and suspended in 2 mL of DF medium containing 3 mM ACC (Merck, Italy) in 12 mL culture tubes. After incubating at 28 °C on an orbital shaker (200 rpm) for 24 h, 1 mL of each culture was centrifuged at 8000 g for 5 min. Aliquots of supernatants (100 μL) were then diluted 1:10 with DF medium, and 200 μL of each diluted supernatant, along with 200 μL of DF+ACC medium to be used as reference, were

mixed to 400 μL of ninhydrin reagent. After 30 min treatment in boiling water bath, three 180 μL aliquots for each isolate and the reference mixtures were transferred to the wells of 96-well polystyrene dishes. The resulting Ruhemann's Purple color was measured by recording the absorbance at 570 nm using the FluoStar Omega microplate reader, and ACC values were determined by a standard curve prepared from ACC solutions ranging between 0.005 and 0.05 mM. Untreated aliquots of cell cultures (180 μL) grown in DF+ACC medium were used for the assessment of cell density by measuring the absorbance at 600 nm. After normalizing residual ACC to cell density (1.5×10^8 cells mL^{-1}), the ACC-utilizing isolates were categorized by calculating the percentage of residual ACC compared with that of the DF+ACC medium without inoculation.

2.6. Biocontrol activity of bacterial isolates

Bacterial strains were tested for their ability to control the growth of the phytopathogenic fungi *Fusarium oxysporum* f.sp. *lentis* (kindly provided by the CNR-ISPA ITEM collection, Bari, Italy) and *Rhizoctonia solani* (kindly provided by Dr. Alessandro Infantino, CREA-DC, Roma, Italy) *in vitro*. Mycelial plugs (6 mm Ø) from 7-day-old cultures of *F. oxysporum* or *R. solani* were placed at the center of Petri plates (90 mm Ø) containing potato dextrose agar (PDA). Bacterial strains were cultured in 2 mL of nutrient broth added with glucose (NBG) on an orbital shaker (200 rpm) for 24 h at 28 °C and streaked (10 μl of suspension, OD_{600nm}= 1) at opposite sides (about 7 cm apart) of PDA plates containing the fungus. Controls were represented by plates containing fungal isolates plugs grown without bacteria. Three replicates were prepared for each bacterial isolate and for fungal controls. After incubation (25 °C for 6 days), the diameters of fungal colonies were measured and the growth inhibition rate (percentage of 1 – dt/dc, where dt is the mean test fungal colony diameter and dc the control one) was calculated for each bacterial strain.

2.7. Bacterial growth assay under osmotic and saline conditions

Strains selected on the basis of biochemical tests were evaluated for their ability to grow under osmotic and saline conditions. The non-permeating osmotic agent polyethylene glycol (PEG), with an average molecular weight of 6000 u, was used to create the osmotic stress conditions (Ansari and Ahmad, 2019). Liquid medium (NBG) containing 10 %, 20 %, 30 %, 40 % and 50 % PEG 6000 was inoculated with 100 μl of a cell suspension of the selected bacterial strains and placed in a shaking incubator set at 28 °C and 120 rpm. As a control, NBG medium without PEG 6000 was utilized. Bacterial growth was verified after 24 h by measuring the OD_{600nm} of a 1:10 dilution of the liquid medium with a spectrophotometer. The spectrophotometer was blanked with a 1:10 dilution of NBG medium with varying concentrations of PEG 6000 without bacteria. In order to assess the bacterial growth under saline stress conditions, 10 mL of bacterial suspension were spot-inoculated on Nutrient with Glucose Agar (NGA) plates containing 2.5 %, 5 %, 7.5 % or 10 % NaCl (Nautiyal et al., 2000). As a control, NGA plates without any further addition of NaCl were used (0.5 % NaCl). Inoculated plates were incubated in the dark at 28 °C, and bacterial growth was verified after three days. For each test, three technical replicates were used.

2.8. Germination assay of Triticum durum seeds inoculated with potential PGP strains under osmotic and saline stress conditions

Four *Triticum durum* genotypes (*i.e.*, Antalis 1242-LIMAGRAIN ITALIA S.P.A., Furio Camillo 110-S.I.S. SOCIETA' ITALIANA SEMENTI, Maestà 1143-CGS SEMENTI S.P.A., and Marco Aurelio 110-S.I.S. SOCIETA' ITALIANA SEMENTI) were surface-sterilized by immersion in 2.5 % v/v NaClO for 10 min, then rinsed six times with sterile distilled water.

Two of the strains previously tested in osmotic and saline stress conditions were selected for this experiment. The bacterial cell suspension was prepared as described previously. Sterile seeds were inoculated by transferring them into the bacterial suspension for 120 min at 180 rpm in the shaking incubator. Non-inoculated seeds were transferred into sterile distilled water solution, all other conditions kept unchanged. After the inoculation, seeds were placed in Petri dishes (30 seeds per treatment) containing PEG 6000 (20 % w/v) or NaCl (1 % w/v) and stored in the dark at 25 °C for 6 days.

Seeds were considered germinated if their root length was at least 5 mm. Germinated seeds were counted after 3, 4 and 5 days of incubation to determine the Germination Index (GI). The GI was calculated as follows:

$$GI = \sum n_d \times d^{-1}$$

where n_d is the number of germinated seeds on day 'd' and d is the number of incubation days (Gupta, 1993). Seeds with a greater GI are considered to be more vigorous.

After six days of incubation, seedlings were collected, and root and shoot length were measured with a ruler to determine the Seed Vigor Index (SVI), a parameter that takes into account both the germination percentage of seeds and the growth of germinated seedlings (Gupta, 1993).

SVI = (root length + shoot length) × germination percentage

In addition, the root-to-shoot ratio (R/S) was calculated as follows:

R/S = (root length) × (shoot length)$^{-1}$.

2.9. Statistical analysis

Data were analysed with the statistical software Past 4.09 (Hammer et al., 2001) and R 4.1.2 (Core Team, 2021; https://www.R-project.org/). The biochemical characterization results were analysed with ANOVA, adopting a probability level of p-value $\leq$ 0.05, and Tukey post-hoc comparison was used. The GI data were analysed with a two proportion z-test by comparing within each genotype, for each stress condition, the non-inoculated with the inoculated, adopting a probability level of p-value $\leq$ 0.05. The different durum wheat genotypes data (SVI and R/S) were analysed separately. After verifying the presence of outliers with the Grubbs' test, outliers were removed and the normality of the data distribution and the homogeneity of variances were verified with the Shapiro-Wilk and the Levene's tests, respectively. Subsequently a two-way ANOVA was used to verify the presence of significant differences among treatments (stress conditions and inoculation). Given the highly significant difference among the stress conditions, a one-way ANOVA was used to test the differences among the inoculation treatments within each stress condition and Tukey post-hoc comparison was used, adopting a probability level of p-value $\leq$ 0.05. If normal distribution and homogeneity of variances were not confirmed, data were analysed with Kruskal-Wallis test and Dunn's post-hoc comparison was used (p-value $\leq$ 0.05).

3. Results

3.1. Isolation and molecular characterization of bacterial endophytes from lentil nodules

A total of 26 bacterial strains were isolated from the surface-sterilized lentil root nodules. The majority of them was Gram positive, and only four strains were Gram negative (10NL1, 10NL2, 17NL2 and 19NL2). The isolates were characterized through 16S rRNA sequencing (providing sequences from 1519 to 1538 bp). The sequences were deposited in the GenBank database (Table 1) and compared with those in the NCBI database by using Blastn. Among the Gram negative isolates, the strains 10NL2, 17NL2 and 19NL2 had the highest similarity with *Pseudomonas koreensis,* while 10NL1 matched with *Acinetobacter calcoaceticus,* which is a potential human pathogen (biological safety level

risk 2). The Gram positive isolates showed the highest similarity with eight genera, namely *Bacillus, Microbacterium, Niallia, Paenarthrobacter, Peribacillus, Plantibacter, Priestia* and *Rossellomorea*. The similarity scores of the five top hits for each isolate are shown in the Supplementary Table 1.

A phylogenetic tree was built with the aligned 16S rRNA sequences of the 26 isolates and of the type strains retrieved from the NCBI database (Fig. 1). The phylogenetic analysis mainly confirmed the Blastn results, with the exception of the strains 11NL2 and 20NL2 that in the Blastn analysis showed identity with two *Peribacillus* species, whilst in the tree they clustered with *Peribacillus frigoritolerans* DSM 8801 and with the strains 18NL3 and 12NL1.

Based on literature search, three strains were discarded for further analyses given their reduced phylogenetic distance from human pathogens (*e.g. Acinetobacter calcoaceticus* 10NL1, accession number OR144218) and for growth difficulties (*Plantibacter* sp. 14NL1 and 17NL3, accession numbers OR144219 and OR144220, respectively).

No band was detected for the tested strains in the PCR analysis performed with primers for *nifH* (data not shown). On the other hand, the PCR using the primer pair for *acdS* encoding gene resulted in a single band of the expected length in the strains 19NL2 and 17NL2 (Supplementary Figure 1), while the strains 9NL3, 10NL2, 11NL2, 11NL4, 17NL1, 18NL2 and 18NL4 led to a band at the expected length, included in a multiple band pattern (Supplementary Fig. 1).

3.2. Biochemical characterization of bacterial isolates and growth assay under osmotic and saline conditions

The biochemical characterization of the selected 23 strains allowed to identify the best IAA producers, namely 11NL3 and 18NL1 producing 6.96 and 6.87 μg IAA × $(10^8$ CFU $mL^{-1})^{-1}$, respectively, and the strains with significantly higher ACC deaminase activity, *i.e.* 9NL2 and 19NL1 with 11 and 10 % of residual ACC, respectively (Table 2). Two strains (*i.e.,* 10 NL2 and 19NL2) showed significantly higher ability to solubilize phosphate, compared to the others, producing a halo of 22±1 and 24±2 mm (mean ± standard deviation; Table 2). For the three *P. koreensis*

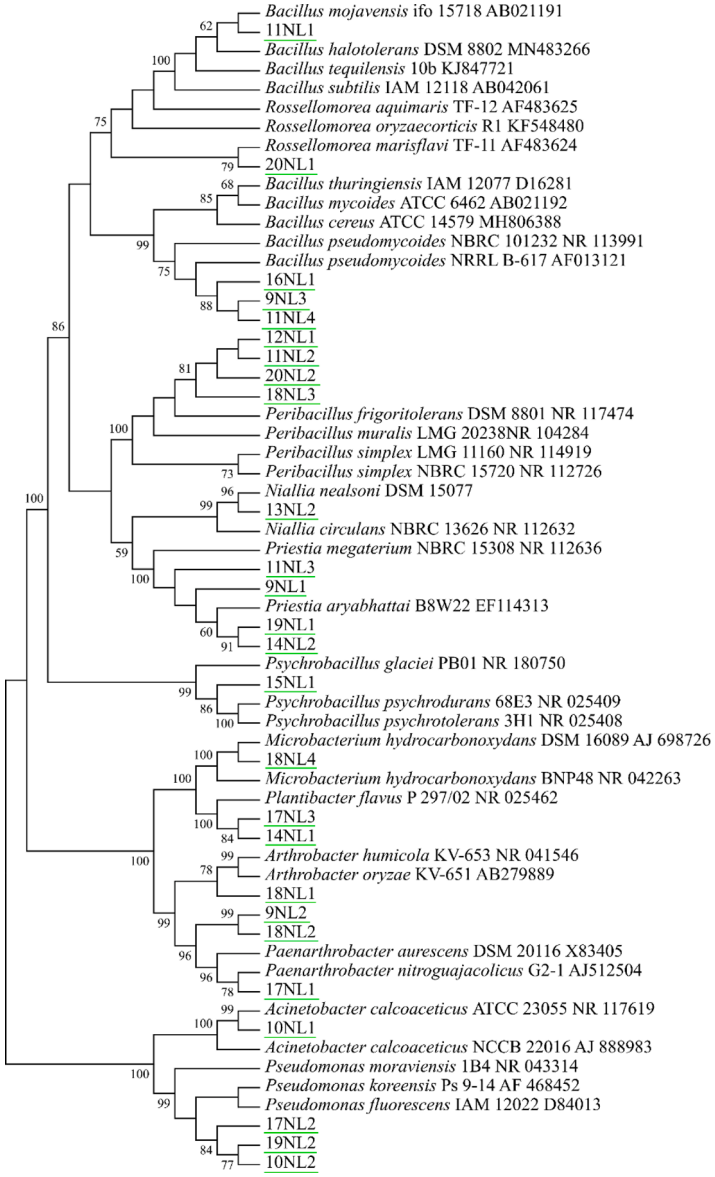

Fig. 1. Maximum Likelihood phylogenetic tree of bacterial 16S rRNA gene sequences. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved 61 nucleotide sequences. There were a total of 1580 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The strains used in this study are underlined in green.

Table 2
Biochemical characterization of the endophytic bacteria isolated from lentil root nodules. For each identified strain (Strain ID) the production of Indole Acetic Acid (μg IAA), the dimension of the inorganic P solubilization halo (P solub; mm) and the percentage of residual 1-aminocyclopropane-1-carboxylic acid (ACC) are reported (± standard deviation). The "nd" (not determined) indicates that the assay could not be successfully be concluded. Different lowercase letters indicate a significant difference ($p < 0.05$) for the ANOVA test, Tukey multiple comparisons.

Strain ID	IAA (μg × (10^8 CFU mL^{-1})$^{-1}$)	P solub (mm)	>residual ACC (%)
Arthrobacter sp. 18NL1	6.87 ± 0.41 a	<5	nd
Bacillus pseudomycoides 9NL3	nd	nd	nd
Bacillus pseudomycoides 16NL1	nd	nd	nd
Bacillus pseudomycoides 11NL4	2.66 ± 0.66 b–d	nd	nd
Bacillus sp. 11NL1	2.23 ± 0.46 c–e	<5	26 % b–e
Microbacterium hydrocarbonoxydans 18NL4	1.71 ± 0.50 c–f	<5	14 % fg
Niallia sp. 13NL2	2.98 ± 0.86 bc	<5	nd
Paenarthrobacter nitroguajacolicus 17NL1	3.95 ± 0.58 b	nd	nd
Paenarthrobacter sp. 9NL2	1.20 ± 0.44 e-g	nd	11 % g
Paenarthrobacter sp. 18NL2	1.43 ± 0.31 d–g	nd	31 % a-c
Peribacillus frigoritolerans 18NL3	0.90 ± 0.35 e–g	<5	34 % a
Peribacillus frigoritolerans 12NL1	0.26 ± 0.09 fg	<5	33 % ab
Peribacillus sp. 20NL2	0.40 ± 0.05 fg	11.3 ± 0.8 de	23 % c–e
Peribacillus sp. 11NL2	2.27 ± 0.86 c–e	9.3 ± 2.0 e	29 % a–d
Priestia aryabhattai 14NL2	0.61 ± 0.16 fg	<5	20 % ef
Priestia aryabhattai 19NL1	1.53 ± 0.61 ɒ-g	<5	10 % g
Priestia megaterium 9NL1	0.48 ± 0.07 fg	13.0 ± 1.1 cde	27 % a–e
Priestia megaterium 11NL3	6.96 ± 0.48 a	15.5 ± 2.0 cd	24 % c–e
Pseudomonas koreensis 19NL2	1.36 ± 0.31 d–g	24.2 ± 2.1 a	20 % ef
Pseudomonas koreensis 17NL2	0.37 ± 0.15 fg	20.3 ± 1.4 ab	nd
Pseudomonas koreensis 10NL2	0.90 ± 0.18 e–g	22.2 ± 1.0 a	nd
Psychrobacillus sp. 15NL1	0.68 ± 0.19 fg	nd	26 % b–e
Rossellomorea marisflavi 20NL1	0.40 ± 0.10 fg	16.1 ± 1.8 bc	23 % de

isolates, values of PSI ranged between 2.6 and 2.8 and those of PE between 102 and 120 %.

The best performing strains in IAA production (11NL3 and 18NL1), ACC deaminase activity (9NL2 and 19NL1) and P solubilization (10NL2 and 19NL2) were selected to further characterization, by assessing their growth in osmotic and saline conditions. On behalf of the osmotic stress tolerance, the best performing strain was 11NL3 that was able to grow in a 50 % PEG concentration (Table 3). The strain 19NL1 showed a reduced growth in 50 % PEG, followed by 19NL2 for which a reduced growth was detected at 40 % PEG concentration. The strain 10NL2 was able to grow at 30 % PEG and the less tolerant strains to osmotic stress were 9NL2 and 18NL1, which were able to grow at a PEG concentration of 20 and 10 %, respectively (Table 3).

Regarding salinity stress, the most tolerant strains were 11NL3 and 19NL1, growing in a NaCl concentration of 10 %, while 9NL2 was able to grow at 7.5 % NaCl, albeit showing reduced growth (Table 3, Supplementary Figure 2). On the contrary, the strains 18NL1, 10NL2 and 19NL2 were less tolerant to salinity stress, ceasing growth above 5 % NaCl concentration (Table 3, Supplementary Figure 2).

3.3. Biocontrol activity of bacterial isolates

None of the tested isolates were able to control the growth of *R. solani*, while 50±2 % growth reduction was detected in *F. oxysporum* cultured with the strain 11NL1. The isolate 11NL3 was not able to reduce *F. oxysporum* growth, although it impaired the aerial mycelium development (Supplementary Table 2).

3.4. Germination assay of Triticum durum seeds inoculated with potential PGP strains under salt and osmotic stress conditions

The two best-performing strains, previously tested in osmotic and saline stress conditions, were selected for this experiment (*Priestia megaterium* 11NL3 and *Priestia aryabhattai* 19NL1). In general, the GI varied from 6.40 to 8.02, from 6.82 to 9.25, from 6.38 to 9.50, and from 5.40 to 9.12 for the Antalis, Furio Camillo, Maestà and Marco Aurelio genotypes, respectively. In particular, the inoculation with 11NL3 and 19NL1 did not affect significantly the vigor of seed germination in water. However, even if not statistically significant, for the Marco Aurelio genotype the inoculation with 11NL3 increased the GI to a greater value (9.12) compared to the value obtained for non-inoculated seeds, *i.e.*, 7.85, whilst the inoculation with 19NL1 decreased the GI value to 5.98 (Fig. 2). In salt stress conditions, the inoculation with the two strains had a negative effect on Furio Camillo genotype, resulting in a lower GI compared to the non-inoculated seeds, in particular, the inoculation with 11NL3 resulted in a significantly lower GI value (6.82; p-value = 0.02797). On the other hand, the two strains had a positive (but not statistically significant) effect on the GI of the Antalis genotype. A different outcome was obtained for the Maestà genotype, in which the inoculation with 19NL1 significantly increased the GI to a value of 9.28 (p-value = 0.03839), while the 11NL3 inoculation slightly decreased it (7.05) compared to the non-inoculated (7.63; Fig. 2). Contrarily to the result in non-stressed conditions, the 11NL3 treatment had a negative effect on the germination vigor of Marco Aurelio seeds in NaCl, significantly decreasing the GI value to 5.40 (p-value = 0.03047). In osmotic stress condition, the inoculation with the two strains had a significantly negative effect (p-values of 0.00625 and 0.01685 for 11NL3 and 19NL1, respectively) and a slightly negative effect on the vigor of seed germination of the Maestà and Furio Camillo genotypes, respectively, while a slightly positive effect was registered for the Antalis and Marco Aurelio genotypes (Fig. 2).

Another indication of vigor was obtained by calculating the seed vigor index (SVI). In the absence of abiotic stress, the inoculation with the two strains did not alter significantly the SVI of the Antalis and Furio Camillo genotypes (ANOVA; Fig. 3). Instead, the inoculation with 19NL1 had a significantly negative effect on the SVI of Marco Aurelio genotype (Kruskal-Wallis p-value = 0.000182), but it significantly increased the SVI value in the Maestà genotype compared to the non-inoculated and to the inoculation with 11NL3 (ANOVA p-value = 0.0006308). In salt stress conditions, no significant difference among treatments in the SVI was obtained for the Antalis and Marco Aurelio genotypes, while in the Furio Camillo and Maestà genotype the SVI was significantly reduced by the inoculation with 11NL3 compared to the uninoculated control (Kruskal-Wallis p-value = 0.004104 and p-value = 0.01941, respectively; Fig. 3). In osmotic stress conditions, no significant difference was found in the SVI among all genotypes, except for the Antalis SVI, that was significantly increased by the inoculation with 11NL3, compared to the

Table 3
Growth of the endophytic bacteria isolated from lentil root nodules under saline and osmotic conditions. The strain identification (Strain ID) is reported as well as the growth under different concentrations of NaCl (0.5, 2.5, 5, 7.5 and 10 %) and of PEG 6000 (0, 10, 20, 30, 40 and 50 %). The + indicates growth, the ± indicates stunted growth and the – indicates absence of growth.

Strain ID	Growth in NaCl					Growth in PEG 6000					
	0.5 %	2.5 %	5 %	7.5 %	10 %	0 %	10 %	20 %	30 %	40 %	50 %
Arthrobacter sp. 18NL1	+	+	+	–	–	+	+	–	–	–	–
Paenarthrobacter sp. 9NL2	+	+	+	±	–	+	+	+	–	–	–
Priestia aryabhattai 19NL1	+	+	+	+	+	+	+	+	+	+	±
Priestia megaterium 11NL3	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas koreensis 19NL2	+	+	±	–	–	+	+	+	+	±	–
Pseudomonas koreensis 10NL2	+	+	±	–	–	+	+	+	±	–	–

Genotype	Condition	Inoculation	Germination Index (GI)
Antalis	NaCl	non-inoculated	6.40
Antalis	NaCl	11NL3	7.45
Antalis	NaCl	19NL1	7.27
Antalis	PEG	non-inoculated	6.85
Antalis	PEG	11NL3	7.52
Antalis	PEG	19NL1	8.02
Antalis	Water	non-inoculated	7.62
Antalis	Water	11NL3	7.70
Antalis	Water	19NL1	7.62
Furio Camillo	NaCl	non-inoculated	8.85
Furio Camillo	NaCl	11NL3	6.82 *
Furio Camillo	NaCl	19NL1	7.78
Furio Camillo	PEG	non-inoculated	9.03
Furio Camillo	PEG	11NL3	8.83
Furio Camillo	PEG	19NL1	8.67
Furio Camillo	Water	non-inoculated	9.23
Furio Camillo	Water	11NL3	9.08
Furio Camillo	Water	19NL1	9.25
Maestà	NaCl	non-inoculated	7.63
Maestà	NaCl	11NL3	7.05
Maestà	NaCl	19NL1	9.28 *
Maestà	PEG	non-inoculated	9.08
Maestà	PEG	11NL3	6.38 *
Maestà	PEG	19NL1	6.88 *
Maestà	Water	non-inoculated	9.50
Maestà	Water	11NL3	9.37
Maestà	Water	19NL1	9.20
Marco Aurelio	NaCl	non-inoculated	7.70
Marco Aurelio	NaCl	11NL3	5.40 *
Marco Aurelio	NaCl	19NL1	7.43
Marco Aurelio	PEG	non-inoculated	6.62
Marco Aurelio	PEG	11NL3	7.53
Marco Aurelio	PEG	19NL1	7.03
Marco Aurelio	Water	non-inoculated	7.85
Marco Aurelio	Water	11NL3	9.12
Marco Aurelio	Water	19NL1	5.98

Scale
9.5
9
8.5
8
7.5
7
6.5
6
5.5

Fig. 2. Heatmap of the Germination Index (GI) of *Triticum durum* seeds. The GI of four different *Triticum durum* genotypes (Antalis, Furio Camillo, Maestà and Marco Aurelio) in non-stressed (Water), salt stress (NaCl) and osmotic stress (PEG) conditions is reported in a color scale. Seeds were inoculated by soaking in a *Priestia megaterium* 11NL3 (11NL3) and *Priestia aryabhattai* 19NL1 (19NL1) bacterial cell suspension (10^8 CFU × ml^{-1}), control seeds were soaked in sterile distilled water (non-inoculated). The GI was calculated as follows:
$GI = \sum n_d \times d^{-1}$
where n_d is the number of germinated seeds on day 'd' and d is the number of incubation days (Gupta, 1993). A greater GI indicates a more vigorous germination of the seeds. Significant differences according to two proportion z-test were indicated with *, with a *p*-value ≤ 0.05).

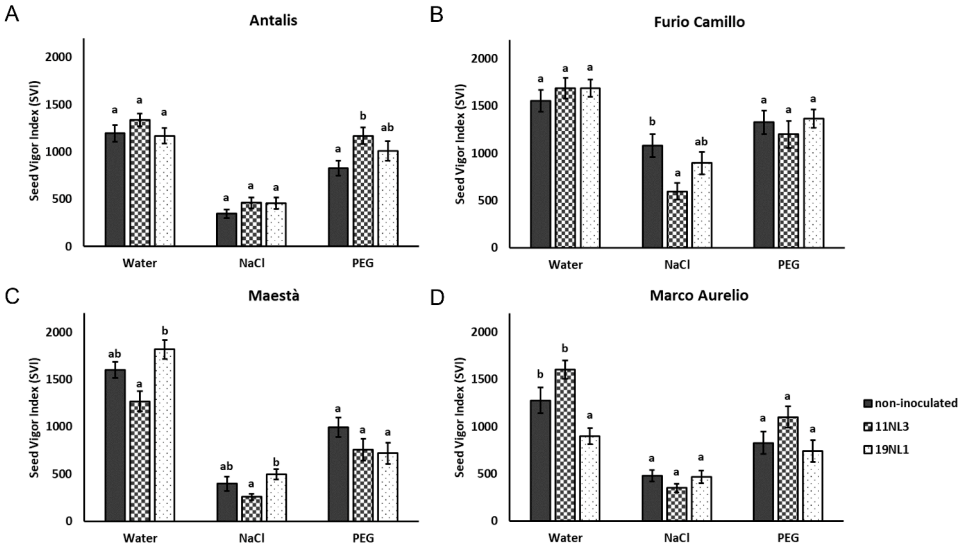

Fig. 3. Seed Vigor Index (SVI) of *Triticum durum* seeds. The histograms show the SVI of four different *Triticum durum* genotypes: Antalis (A), Furio Camillo (B), Maestà (C) and Marco Aurelio (D) in non-stressed (Water), salt stress (NaCl) and osmotic stress (PEG) conditions. Seeds were inoculated by soaking in a *Priestia megaterium* 11NL3 (11NL3) and *Priestia aryabhattai* 19NL1 (19NL1) bacterial cell suspension (10^8 CFU × ml^{-1}), control seeds were soaked in sterile distilled water (non-inoculated). The SVI was calculated as follows
SVI = (root length + shoot length) × germination percentage.
Different lowercase letters indicate significant differences among the treatments (non-inoculated, 11NL3 and 19NL1) within the stress condition (Water, NaCl and PEG) (one-way ANOVA or Kruskal-Wallis test *p*-value ≤ 0.05, Tukey's or Dunn's post-hoc comparisons). Error bars indicate standard error.

uninoculated control (Kruskal-Wallis *p*-value = 0.02522; Fig. 3). In absence of stress conditions, the root to shoot ratio (R/S) was around or slightly below one in all the genotypes, indicating that the root had a similar length to the shoot. No significant difference in the R/S was found for the Antalis and Furio Camillo genotypes, when comparing the different treatments (ANOVA). Instead, the inoculation with 19NL1 significantly increased the R/S in the Maestà genotype compared to the other treatments (ANOVA *p*-value = 0.004895; Fig. 4). On the other hand, the inoculation with 19NL1 significantly reduced the R/S in the Marco Aurelio genotype compared to the non-inoculated seeds (ANOVA *p*-value = 0.0309; Fig. 4). In general, the salt and osmotic stress induced a major development of the root compared to the shoot in all the considered genotypes, resulting in a higher value of R/S. In salt stress conditions, the R/S was significantly higher in the Antalis genotype when the seeds were inoculated with 19NL1, compared to the non-inoculated ones (Kruskal-Wallis *p*-value = 0.005871; Fig. 4). In contrast, the R/S was significantly reduced when the Marco Aurelio seeds were inoculated with 11NL3, compared to the non-inoculated ones

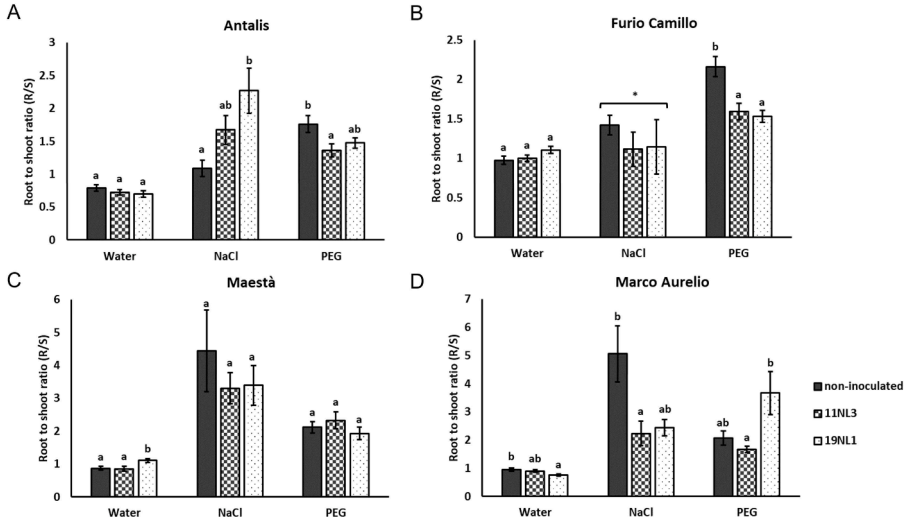

Fig. 4. Root-to-shoot ratio (R/S) of *Triticum durum* seeds. The histograms show the R/S of four different *Triticum durum* genotypes: Antalis (A), Furio Camillo (B), Maestà (C) and Marco Aurelio (D) in non-stressed (Water), salt stress (NaCl) and osmotic stress (PEG) conditions. Seeds were inoculated by soaking in a *Priestia megaterium* 11NL3 (11NL3) and *Priestia aryabhattai* 19NL1 (19NL1) bacterial cell suspension (10^8 CFU × ml^{-1}), control seeds were soaked in sterile distilled water (non-inoculated). The R/S was calculated as follows:
R/S= (root length) × $(\text{shoot length})^{-1}$.
Different lowercase letters indicate significant differences among the treatments (non-inoculated, 11NL3 and 19NL1) within the stress condition (Water, NaCl and PEG) (one-way ANOVA or Kruskal-Wallis *p*-value ≤ 0.05, Tukey's or Dunn's post-hoc comparisons). Significant differences among treatments were indicated with *. Error bars indicate standard error.

(Kruskal-Wallis p-value = 0.02821; Fig. 4), and, even if not statistically significant compared to the non-inoculated, also the seeds treated with 19NL1 showed a reduction in the R/S. The Furio Camillo genotype was characterized by a significant difference among the treatments in the salt stressed seeds (Kruskal-Wallis p-value = 0.03383; Fig. 4), even if the Dunn's post-hoc test could not identify the differences among treatments, it was possible to note that the R/S was lower in the inoculated seeds compared to the non-inoculated. Even if not statistically significant, it was possible to identify a trend in the R/S of the salt-stressed Maestà seeds that showed a reduced value of the inoculated compared with the non-inoculated ones (Fig. 4). In osmotic stress conditions, the R/S of the inoculated seeds was significantly reduced in the Furio Camillo genotype, compared to the non-inoculated ones (Kruskal-Wallis p-value = 0.01203; Fig. 4). A similar trend was observed in the Antalis genotype, in which the R/S was lower in the inoculated seeds, compared to the non-inoculated ones; in particular, the seeds inoculated with 11NL3 showed a significantly lower R/S compared to the non-inoculated ones (Kruskal-Wallis p-value = 0.001846; Fig. 4). A higher R/S was measured for the 19NL1-inoculated seeds in Marco Aurelio genotype compared to the seeds inoculated with 11NL3, however, this difference was not significant compared to the non-inoculated seeds (Kruskal-Wallis p-value = 0.004641; (Fig. 4). No statistically significant difference was found in the R/S of the Maestà seeds inoculated and non-inoculated in osmotic stress conditions (Fig. 4).

4. Discussion

Legume nodules harbor not only nitrogen fixing rhizobia, but also PGP bacteria that improve nodule formation and increase nutrient availability in the root zone (Rajendran et al., 2012; Kumawat et al., 2019; Preyanga et al., 2021). This study presents the identification and characterization of new endophytic bacterial strains isolated from root nodules of lentil grown in an open field intercropping experiment in the Southern Italy. The 26 bacterial isolates were identified using 16S sequencing, a Blastn search, and a phylogenetic tree. These were belonging to the orders Bacillales, Pseudomonadales and Micrococcales, which was comparable to the findings of Debnath et al. (2023) that isolated endophytic bacteria belonging to the genera *Enterobacter, Serratia, Bacillus* and *Pseudomonas* from lentil nodules in India. The phylogenetic tree obtained with the 16S sequences mainly confirmed the Blastn results. However, in the phylogenetic tree two strains identified as *Peribacillus frigoritolerans* in the Blastn analysis, namely 12NL1 and 18NL3, clustered together with other two here characterized strains (*i.e.*, 11NL2 and 20NL2) and with the strains *Peribacillus muralis* LMG 20238 and *Peribacillus frigoritolerans* DSM 8801 with a Bootstrap value of 100. In a recent study, *Peribacillus frigoritolerans*, previously assigned to the *Brevibacterium* genus, was reclassified and appeared closely related to *Peribacillus muralis* and *Peribacillus simplex* (Montecillo and Bae, 2022). It is possible that the strains 12NL1, 18NL3, 11NL2 and 20NL2 cannot be unequivocally identified at the species level only with the 16S locus, but other loci may be employed to clarify their taxonomic assignment.

To further characterize the strains, the presence of *nifH* gene was assessed by PCR. This gene is widely used to test the nitrogen fixation ability of microorganism (Collavino et al., 2014), however the result was negative for all the strains. This molecular result may indicate either that the gene is not present in the strain genomes, or that the used primer pairs were not optimal for our strains. Indeed, the used primers were designed and tested *in silico*, and even if this pair was the best performing, its efficacy may not be the same when tested *in vitro* and may vary according to the different species (Wang et al., 2022). Another important trait in a potential PGP strain is the presence of the *acdS* gene, encoding the enzyme ACC deaminase. This enzyme reduces the quantity of ACC that can be converted into ethylene by the plant. By controlling ethylene production in stressed plants, ACC deaminase-producing bacteria can increase plant yield (Jaemsaeng et al., 2018; Yoolong et al., 2019). Differently from the *nifH* primers, the primer pair for *acdS* gene resulted in a single and multiple band pattern for several strains. This result was in agreement with that of Blaha et al. (2006) that found the expected band with other bands in some strains. In this case, the biochemical assay for residual ACC partly confirmed the molecular result (*e.g.*, 19NL2 single band and 18NL4 multiple band pattern). Unfortunately, most of the isolates showing positive results to the PCR assay could not be tested for ACC deaminase activity due to their inability to grow in the liquid medium. Interestingly, the strains with the highest ACC deaminase activity were *Paenarthrobacter* sp. 9NL2 and *Priestia aryabhattai* 19NL1. Formerly known as *Bacillus aryabhattai* (Gupta et al., 2020), *Priestia aryabhattai* has already been reported for its ACC deaminase ability, ameliorating salt stress negative effects on canola seeds (Siddikee et al., 2015).

Two additional relevant PGP traits, namely IAA production and P solubilization, were also investigated. IAA is the main auxin in plants and, when present in the right concentrations, it promotes stem elongation, tropism, adventitious root growth, and cell division, among other functions (Aloni et al., 2006). The strains *Priestia megaterium* 11NL3 and *Arthrobacter* sp. 18NL1 were the best IAA producers, with 300 and 295 μg IAA per mL of culture, respectively. Indeed, *Priestia megaterium*, formerly known as *Bacillus megaterium* (Gupta et al., 2020), has already been reported as a plant endophyte with growth promoting traits, including IAA production (Chakraborty et al., 2006; Feng et al., 2017). The ability of the genus *Arthrobacter* to produce IAA, promoting plant growth, is widely reported and frequently associated with a reduction in plant stress when *Arthrobacter* is used as inoculant (Kumar et al., 2014; Safdarian et al., 2019).

One important macronutrient that is crucial for the growth and development of plants is P. Due to its low solubility and rate of fixation, P is poorly available for plants in agricultural soil (Zhu et al., 2018). According to various works, the genus *Pseudomonas* is one of the most efficient phosphate solubilizers (Hernández-Salmerón et al., 2016; Kwak et al., 2016; Liu et al., 2019). In accordance, in this work the best results were consistently obtained for the strains identified as *Pseudomonas koreensis* (10NL2, 17NL2 and 19NL2), a species previously reported as an efficient solubilizer of both inorganic and organic P, able to promote plant growth and health (Srivastava et al., 2019; Singh et al., 2023).

No significant biocontrol activity could be detected for all the strains although one, namely *Bacillus* sp. 11NL1, reduced *F. oxysporum* radial growth in plate assays. Previous studies showed that even rhizospheric strains with confirmed PGP traits may lack the ability to control the growth of phytopathogenic fungi in culture, depending on the identity of competing fungi and on the composition of the used growth medium, which may affect the interaction outcomes by modulating antifungal molecules (Bach et al., 2016; Mezghanni et al., 2012). Even if PDA is a standard medium used to test biocontrol activity toward fungi, it is possible that other specifically formulated media may reveal antifungal activity in the tested bacterial strains.

The six best performing strains in biochemical tests for PGP traits were selected to test their tolerance to osmotic and saline stress conditions. *Priestia megaterium* 11NL3 and *Priestia aryabhattai* 19NL1 showed strong tolerance to osmotic and salt stress, being able to grow in a concentration of 50 % PEG and 10 % NaCl, respectively. To our knowledge, few studies focused on salinity and osmotic stress tolerance of bacteria isolated from legume root nodules of plants growing in non-saline soils (El Attar et al., 2019; Hnini et al., 2022). However, the utilization of halotolerant and osmotolerant PGPB to reduce the negative impact of soil salinity and drought on plant development is well documented. Tolerance to salinity was improved in pea plants by reducing ethylene levels with the ACC deaminase-producing rhizobacteria *Arthrobacter protophormiae* (SA3) (Barnawal et al., 2014). Through the production of a high amount of IAA, among other PGP traits, the beneficial strain *Bacillus mojavensis* I4 increased the root system development of wheat, increasing plant nutrient uptake in saline conditions (Ghazala et al., 2023). Since in drought situations the root development is critical in order to reach areas of moisture in the surrounding soil, the

use of strains producing IAA able to stimulate the development of the host roots by increasing lateral and adventitious root formation (He et al., 2021; Dixit et al., 2022) might reduce plant stress damage. Indeed, *Bacillus* sp. WM13–24 and *Pseudomonas* sp. M30–35 strains, able to produce IAA, improved root development and enhanced the growth of perennial ryegrass in drought conditions (He et al., 2021).

Since PGPB were already reported to cross-colonize different plant species in intercropping (cereal-legumes intercropping) (Vora et al., 2021), we tested the effect of the inoculation of the two osmotolerant and halotolerant strains isolated from lentil on four different durum wheat genotypes. There is widespread agreement that the plant genotype may strongly influence the plant-bacterial interaction and the positive effects of PGPB in stress conditions (Kazi et al., 2016; Valente et al., 2020). Indeed, the results obtained for each of the tested genotype were different, and it was not possible to identify a common trend in the results for the different genotypes and stress treatments. For instance, the GI of Marco Aurelio genotype was positively affected by the inoculation with *P. megaterium* 11NL3 in absence of stress, but in saline condition the same inoculum negatively affected the GI value, while *P. aryabhattai* 19NL1 improved the GI of Maestà genotype exposed to salt stress although no or negative responses were detected in water or under osmotic stress. Similarly, the SVI was significantly increased for the Antalis genotype inoculated with *P. megaterium* 11NL3 in osmotic stress conditions, while the same inoculum significantly decreased the SVI for the Furio Camillo genotype in saline stress conditions. High values of the R/S ratio are generally correlated with a high resistance to drought stress in wheat (Bacher et al., 2022). Here, an improvement in the R/S under osmotic stress was registered only for the Marco Aurelio genotype inoculated with *P. aryabhattai* 19NL1. It is of note that the same isolate enhanced R/S values in Antalis genotype under NaCl stress, suggesting a multi-stress reducer role for this strain. For the other genotypes, no significant improvement in the R/S was induced by the inoculation with the two selected strains, with the exception of Furio Camillo, which responded negatively to the bacterial inoculation.

The absence of a common trend in responses to microbial inoculation among different plant genotypes, detected in durum wheat in this work, is often reported, as the variability in genotype-specific gene expression of hosts may greatly affect the outcome of the interaction between cultivar x growth regime x microbial inoculation (Moutia et al., 2010). The combinations of wheat genotypes and potential PGP strains may not be optimal, and the complex interplay of the three factors may lead to inconsistent results in plant growth promotion effects among genotypes (Sasaki et al., 2010; Sharma et al., 2022). In addition, it has been reported that modern wheat varieties interact poorly with rhizobacteria, compared to the ancient ones (Valente et al., 2020) and the durum wheat genotypes used in this study are recent varieties. Indeed, ancient wheat genotypes harbor higher diversity of rhizosphere bacterial community than modern cultivars, that tend to attract mainly pseudomonads (Germida and Siciliano, 2001).

Moreover, seed germination phase may not be the phenological stage at which the bacterial inocula exert their major positive influence on the plants. Until one decade ago, the effect of crop phenological stages on the effectiveness and success of PGPR inoculation was overlooked, even if it is widely known that plants shape their associated microbiome according to their growth stage, recruiting beneficial microbes when needed through secretion of organic acids (Rudrappa et al., 2008; Bashan et al., 2014; Vora et al., 2021). Chen et al. (2019) demonstrated that wheat growth stage strongly influenced bacterial community composition in the rhizosphere and roots and Stoll et al. (2021) recently reported in different horticultural crops the importance to identify the best inoculation strategy, including the growth stage of the crop, to maximize the beneficial effects of the PGP strains. Similarly, different inoculation strategies were tested applying bacteria inocula at diverse phenological stages of wheat, highlighting the improved results obtained with a double bacterial application at sowing and tillering stages (Pagnani et al., 2020).

5. Conclusions

The use of PGPR is a sustainable approach for improving yields of crops and increase their resilience to environmental factors that are becoming more challenging as a result of climate change. In this study, bacteria isolated from root nodules of lentil plants grown in intercropping with durum wheat, characterized by molecular and biochemical methods, were selected for their tolerance to osmotic and saline stress and tested on four durum wheat genotypes assessing their effect on seed germination, vigor and root to shoot ratio in different stress conditions. The selected strains demonstrated interesting PGP traits using molecular and biochemical assays, although *in vitro* germination assays showed that their effect on durum wheat seeds varied depending on the type of stress and on the different durum wheat genotypes. Further studies are needed to test the selected bacterial strains at different plant growth stages in mesocosms, possibly comparing ancient wheat genotypes with modern varieties.

Availability of data and materials

The strains used in this study were deposited and are publicly available at the Agro-Food Microbial Culture Collection of the Institute of Sciences of Food Production (Italian National Research Council).

The 16S rRNA gene sequences were deposited in the GenBank database with the accession numbers from OQ686722 to OQ686744 and from OR144218 to OR144220.

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CRediT authorship contribution statement

Francesca Brescia: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Fabiano Sillo:** Investigation, Formal analysis, Writing – review & editing. **Raffaella Balestrini:** Conceptualization, Investigation, Formal analysis, Supervision, Writing – review & editing. **Cristiana Sbrana:** Investigation, Formal analysis, Supervision, Writing – review & editing. **Elisa Zampieri:** Investigation, Formal analysis, Writing – review & editing, Supervision.

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

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2023.100205.

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