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Application of endophytic bacteria isolated from root nodules of Sulla aculeolata L. and Rhizobium sullae KS6 consortium in the growth of legume Sulla flexuosa L

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Abstract

Legume plants rely upon multipartite interactions between rhizobia and bacterial endophytes within root nodules to facilitate plant growth. This study sought to isolate and identify indigenous endophytic bacteria from root nodules of *Sulla aculeolata* L. in Northeast Morocco. Based on their tri-calcium phosphate (TCP) solubilization capacity, five endophytes were chosen for further evaluation of their plant growth activities. All isolates were hydrogen cyanide (HCN) and siderophore producers, while only BCH24 tested positive for ACC deaminase activity. Indole-3-acetic acid (IAA) synthesis ranged from 1.27 mg L^{-1} to 2.89 mg L^{-1}, with soluble phosphate concentrations between 7.99 mg L^{-1} and 110.58 mg L^{-1}. Additionally, all the endophytes were able to produce more than two lytic enzymes. The 16S rRNA gene sequencing identified the five isolates as *Enterobacter* sp (BCH13, BCH2), *Pseudomonas* sp (BCH16, BCH24), and *Serratia* sp (BCH10). The strains inhibited the growth of three phytopathogenic fungi, with BCH13 exhibiting the highest rate against *Aspergillus ochraceus* (45%), followed by BCH24 against *Fusarium oxysporum* (40%) and *Botrytis cinerea* (35%), respectively. *In vivo* inoculation of halotolerant strains *Enterobacter hormaechei* (BCH13) and *Pseudomonas moraviensis* (BCH16) under chamber conditions revealed that co-inoculation with *Rhizobium sullae* KS6 improved plant development compared to single inoculation, making it a promising eco-friendly bio-inoculant for legume *Sulla flexuosa* L. production.

Introduction

The Mediterranean regions are expected to be particularly vulnerable due to the impacts of climate change such as high temperatures, sparse precipitation, and long dry seasons (Muluneh 2021). These pressures, combined with increasing anthropogenic influence, have led to the loss and extinction of numerous pastoral and forage species, as well as accelerated soil degradation and changes in soil microbial communities (Brígido et al. 2019; Martínez Alcántara et al. 2020). The biodiversity of Northeast Morocco is widely renowned, as it is home to numerous endemic species, including forage legumes. Of particular importance, the native *Hedysarum* spp., which should be preserved and valued as they are well-adapted to Mediterranean climatic conditions, and therefore, can serve as potential fodder legumes (Nichols and Norton 2016; Chiboub et al. 2016). The main species of *Hedysarum* sp reported in Northern Morocco is *Sulla flexuosa* L., its introduction into the region holds significant economic interest, serving as a valuable forage resource and as a protective factor against erosion. *S. flexuosa* is especially noteworthy, being an excellent example of a species adapted to Mediterranean environments and providing a variety of uses, including its application as forage (Elyemlahi et al. 2019; Zirmi-Zembri 2021). Unfortunately, it is classified as endangered due to the difficulties encountered with its uncultivated foraging habits (Groom 2010).To improve the heritage of this spontaneous forage species in the Mediterranean region, it is important to promote more sustainable agricultural practices. This could include the use of biological inoculants, particularly those isolated from the same genus. The benefits of efficient symbiosis between legumes and nitrogen-fixing bacteria in agricultural output are well known, these advantages stem from the bacteria's capacity to fix substantial amounts of atmospheric nitrogen, enabling them to survive in nitrogen-depleted soils without the need for N fertilizers while also raising the nitrogen content of the soil (Cheng et al. 2023; Álvarez-Aragón et al. 2023). Native legumes may not only be adapted to Mediterranean conditions due to their genetics but also due to the establishment of beneficial bacterial associations with soil microbes (Afzal et al. 2019). Consequently, native legumes are likely to represent a valuable reservoir of endophytic bacteria with potential for biotechnological and agronomic applications. Bacterial endophytes colonize plant tissues without causing visible pathogenic symptoms, and they can produce enzymes, phytohormones, nutrient mobilization and translocation processes like phosphate solubilization, nitrogen fixation, and ammonia generation, which is deemed advantageous for plants under extreme abiotic stresses like drought and salinity (Chaudhary et al. 2022; Ali et al. 2022). Consequently, co-inoculation of legumes with rhizobia and other PGP traits presenting endophytes such as *Azotobacter*, *Pseudomonas*, *Bacillus*, *Erwinia*, *Serratia*, and *Enterobacter* can serve as an alternative measure to reduce abiotic stresses in plants (Shrivastava et al. 2018; Sepúlveda-Caamaño et al. 2018; del Barrio-Duque et al. 2019; Rawat et al. 2021). In a recent study, Benjelloun et al. 2021 reported that co-inoculation of chickpeas with phosphate solubilizing bacteria and *Mesorhizobium ciceri* in P-deficient soils in dry areas in Mediterranean regions resulted in a significant increase in nodulation, crop yield, biomass production, N, P, and protein content in grains, this outcome was significantly better than of plants with a single inoculation. According to Molina-Romero et al. 2017, a bacterial consortium containing four compatible and desiccation-tolerant strains was formulated and evaluated for its effects on the growth of blue maize. The results showed that inoculation with the consortium increased the shoot and root dry weight, plant height, and plant diameter were equally promoted compared to the non-inoculated control samples or mono-inoculation treatments. The recent

increased attention towards sustainable agriculture has led to the focus on the formation of novel bacterial consortiums presenting a profile adequate for plant growth promotion. This particular study was carried out to assess the potential of nodule endophytic bacterial strains isolated from *Sulla aculeolata* L. and *Rhizobium sullae* to enhance the growth of a model legume *Sulla flexuosa* L. (Tangier ecotype), along with investigating their ability to solubilize inorganic phosphate, identifying additional growth-promoting features, as well as exploring their antagonistic activity and salt tolerance.

Materials and Methods

Study area and plant material

An investigation of wild/endemic legume plants occurring in the Mediterranean environments was conducted in Beni Chiker, Northeastern Morocco located at 35°16'14"N and 003°00'46"W. This study was primarily focused on the legume species *Sulla aculeolata* L.. Chemical analysis of soil conducted at the National Center of Scientific and Technical Research (CNRST) in Rabat indicated that the soil is of calcareous nature, mainly composed of clay (63.83%), and contains 33.592% total $CaCO_3$, with 5.115 ppm of P_2O_5 and has a pH of 8.

Nodules collection and bacterial isolation

Following surface-sterilization of healthy nodules collected from the root of *Sulla aculeolate* L. plants, bacterial endophytes were isolated as described by Vincent (1970). To confirm the presence of endophytic bacteria, uncrushed surface sterilized nodules were cultured on Yeast Extract Mannitol (YEM) agar; no bacterial growth was observed after 7 days of incubation at 28°C. The pure isolates were then stored at -20°C in 25% (*v/v*) sterile glycerol for future use.

Inorganic phosphate solubilization

The isolated endophytic strains were screened for their capacity to dissolve tri-calcium phosphate ($Ca_3(PO_4)_2$) using the Pikovskaya agar plates (Pikovskaya,1948). Strains presenting a clear halo zone around their colonies were considered phosphate solubilizing bacteria (PSB) and were selected for further study. The selected isolates were then inoculated into PVK's broth, with un-inoculated broth serving as a reference. The quantity of solubilized phosphorus was calculated using the Ames method (Ames,1966), and the pH was determined using a pH meter (pH-221 Biobase).

Molecular characterization

Total DNA was extracted from the selected phosphate- solubilizing bacterial isolates using the phenol/chloroform method (Guerrouj et al. 2013). The DNA concentration was then adjusted to 100 ng/µl with a Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000). The nearly full-length 16S rRNA genes were amplified and sequenced using the universal primers fd1 and rd1 (Weisburg et al. 1991) on a 3130Xl automatic sequencer at the National Centre for Scientific and Technical Research (CNRST) in Rabat (Morocco). The sequences obtained were then assembled using the sequence alignment-editing program Bioedit (7.0.5.3), verified manually and compared to those from GenBank using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi/). Visualization and alignment of the sequence data were done using the Clustal W software. Using the Molecular Evolutionary Genetics Analysis (MEGA), a phylogenetic tree was constructed with the neighbor-joining method and a bootstrap analysis of 1000 resamplings.

Hydrogen cyanide (HCN) production

The efficacy of HCN production by bacterial cultures was assessed by inoculating bacterial isolates on plates containing YEM agar modified with 4.4 g L^{-1} glycine and overlaying a piece of Whatman filter paper No. 2 soaked in 0.5% of picric acid solution (Bakker and Schippers 1987). A change in color from yellow to orange was used as an indicator of successful HCN production.

Siderophores production

The method proposed by Schwyn and Neilands (1987) for quantifying the synthesis of siderophores by selected bacterial endophytes involves incubating the CAS solution and culture bacteria for 30 minutes in the dark. Pal and Gokarn (2010) further refined the method by providing a formula for calculating the percentage of siderophore units. This formula takes into account the absorbance of the reference (CAS reagent) and the absorbance of the sample at 630 nm and is expressed as : $\%\mathrm{ofsiderophoreunits} = \frac{\mathrm{Ar}-\mathrm{As}}{\mathrm{Ar}} \times 100$, where Ar and As represent the absorbance values of the reference and the sample, respectively.

Indole acetic acid (IAA) production

Gordon and Weber (1951) conducted a qualitative approach to investigate the synthesis of indole acetic acid (IAA) via bacterial culture in SMS (Salt Medium Sucrose) containing 0.05% of tryptophan. The material was incubated at 28°C for two days and then centrifuged for three minutes at 13000 rpm. Subsequently, 1 mL of the supernatant was mixed with 2 mL of Salkowski reagent and incubated at room temperature for 20 minutes before measuring the optical density at 535 nm. A standard IAA solution was used to determine the concentration of IAA in the supernatant.

Aminocyclopropane-1-carboxylate (ACC) deaminase activity

The presence of ACC deaminase can be determined by utilizing the (Jacobson et al. 1994) method, which involves comparing the growth rates of bacterial isolates cultured in the presence of two nitrogen sources: ACC and ammonium sulfate $(NH_4)_2SO_4$, as well as a mineral source, magnesium sulfate ($MgSO_4.7H_2O$). After 48 hours, the optical density at 600 nm is measured. Positive ACC production is indicated when isolates display an OD value greater than that of the $MgSO_4.7H_2O$ solution.

Extracellular enzymes production

Bacterial isolates' capacity for cellulose degradation was assessed by streaking bacterial inoculants on cellulose congo-red agar media, as described by (Gupta et al. 2012). Amylase activity was determined using a soluble starch-yeast extract medium (Kammoun et al. 2008; Tsai et al. 2007). Protease activity was evaluated by measuring the clear zone in skimmed milk agar (Chaiharn et al. 2008). Urease activity was evaluated according to the protocol outlined by (Maheshwari et al. 2010). Meanwhile, the presence of a clear halo around the colonies on the pectin medium was indicative of pectinolytic activity (Presence of pectinase), as per the method described by (Cattelan et al. 1999).

Antagonism activity

The antifungal impact of the endophytic isolates was examined on the growth of two fungal laboratory cultures, *Aspergillus ochraceus* and *Fusarium oxysporum*, previously isolated and identified by (El Aaraj et al. 2015), and *Botrytis cinerea*, isolated from cultured strawberries in Larache, Morocco. The antifungal activity was tested using a dual culture assay (Thaware et al. 2016). For each fungal species, a 5 mm diameter disk of the tested fungus was placed at the center of a plate containing Potato dextrose agar (PDA) medium, and bacterial cultures were streaked on both sides within a 30 mm distance from the fungal disk. The plates were incubated at 25°C for 7 days and the inhibitory activity was calculated using the following formula: $\left(\frac{\mathrm{Dc}-\mathrm{Dt}}{\mathrm{Dc}}\right) \times 100$, where Dc is the diameter of the control and Dt is the diameter of the treatment (Tiru et al. 2013). Control experiments were performed without bacteria.

Halotolerance essay

To test the salt-tolerance properties of the five selected endophytic bacteria, YEM liquid medium supplemented with various concentrations of NaCl (*w/v*) was used: 2.50% (427 mM), 5% (854 mM), 7.50% (1.30 M). Fresh bacterial cultures were incubated for 48 hours at 28 ± 2°C, and the growth was evaluated by determining the absorbance at OD_{600nm}, each experiment was carried out in replicates for accuracy. YEM liquid medium containing 0.05% (8.54 mM) NaCl (*w/v*) served as the control test.

Inoculation test

Two isolates, BCH16 and BCH13, were selected based on their PGP activities and halotolerant capacity to determine their impact on the growth of *Sulla flexuosa* L. plant, the experiment was conducted under culture chamber conditions (25/26°C;16/8h day/night). A pot experiment was performed to analyze the single and combined effects of endophytes strains and *Rhizobium sullae* KS6 strain (MT776721.1) isolated and characterized by El Yemlahi et al. 2022. The seeds collected from legume plants in the Tangier Province were disinfected by continuous agitation in 75% ethanol for 20 seconds, followed by a one-minute agitation in 95% sulfuric acid. This was then followed by successive washing (X7) with sterilized water. Each pot (25cm x 22cm x 15cm), filled with sterilized sand, was planted with four seeds and each seed was inoculated directly with 1 ml of bacterial culture grown in liquid YEM medium. Un-inoculated pots were used as controls; pots with soluble nitrogen served as positive assay whilst ones deprived of mineral nitrogen were designated as negative control. A chlorophyll meter (SPAD-502) was used to determine the SPAD value. Plants were harvested after 90 days and data were taken on the shoot, root dry weight, root length, as well as fresh weight and shoot.

Statistical analysis

Analysis of the PGP features of the isolates, antifungal activity, and the effect of bacterial inoculation on plants was conducted using ANOVA and the Fisher's protected LSD test ($p < 0.05$) with Statgraphics Plus version 4.0. The experiments were conducted with three or four replicates.

Results

As shown in Table 1, from 21 endophytic isolates, 5 strains were selected based on their ability to exhibit significant phosphate solubilizing capacity with halo diameters ranging from 0.5 cm to 0.6 cm. Subsequently, molecular identification was performed on all selected isolates.

Table 1
Plant growth-promoting abilities of representatives of root-nodule bacteria isolated from *Sulla aculeolata* L. plant.

Strains	Closetest	D.H (cm)	HCN	IAA (mg L^{-1})	Sid %	P (mg L^{-1})	pH	ACC	Cellu	Amy	Prot	Ure	Pec
BCH13	Enterobacter bugandensis	0.6	+++	2.81^{b}	53.8^{b}	7.99^{a}	5.59^{bc}	-	-	-	+	+	+
BCH10	Serratia plymuthica	0.5	+++	2.89^{c}	60.04^{c}	31.05^{b}	5.87^{c}	-	+	+	+	+	-
BCH24	Pseudomonas moraviensis	0.5	+++	2.58^{bc}	57.69^{bc}	110.58^{c}	5.64^{bc}	+	-	-	+	+	+
BCH2	Enterobacter hormaechei	0.6	+++	1.27^{a}	47.68^{a}	34.08^{b}	4.04^{a}	-	+	+	-	+	+
BCH16	Pseudomonas moraviensis	0.5	+++	1.51^{ab}	69.98^{d}	27.33^{b}	5.52^{b}	-	-	+	-	+	-

D.H, Diameter of solubilization halos; HCN, Hydrogen cyanide; IAA, Concentration of indole acetic acid; Sid, Siderophores; P, Concentration of solubilized Phosphate; ACC, 1−aminocyclopropane−1−carboxylate deaminase; +, Positive; −, Negative; Cell, Cellulase; Amy, Amylase; Prot, Protease; Ure, Urease. Pec, Pectinase. Values in the same column followed by the same superscript letter were not significantly different $p < 0.05$ (Fisher's least significant difference (LSD) test).

16S rRNA gene sequencing of selected endophytic strains

The molecular characterization of the phosphate-solubilizing bacteria associated with *Sulla aculeolata* L. was of interest to gain further insight into the diversity of bacteria present in *Sulla* sp. root nodules. Sequence alignments of the nearly complete 16S rRNA

gene revealed that the strains were highly similar (98–99% similarity) to members of the Gamma-Proteobacteria class, which include *Enterobacter* sp. (BCH2, BCH13), *Pseudomonas* sp. (BCH24, BCH16), and *Serratia* sp. (BCH10) (Fig. 1).

Plant-growth-promoting traits of endophytic bacteria

The quantification of TCP solubilization by the tested strains in a PVK liquid medium was assessed. The highest rate of P solubilization was observed with *Pseudomonas moraviensis* BCH24 (110.58 mg L^{-1}), followed by *Enterobacter* sp. BCH2 (34.08 mg L^{-1}), and *Serratia plymuthica* BCH10 (31.05 mg L^{-1}). The lowest concentration of solubilized P was recorded by BCH13 strains (7.99 mg L^{-1}). A negative correlation between the available P and pH values was observed (Table 1). Additionally, all selected endophytes were positive for IAA, siderophore, and HCN production. IAA production rates ranged from 1.27 mg L^{-1} (*Pseudomonas* sp. BCH16) to 2.89 mg L^{-1} (*Serratia plymuthica* BCH10) in the presence of L-tryptophan as a precursor. The percentage of siderophore units released varied from 47.68% (*Enterobacter* sp. BCH2) to 69.98% (*Pseudomonas* sp. BCH16). The presence of ACC deaminase activity was investigated by supplementing the bacterial medium with ACC as the sole nitrogen source. Only *Pseudomonas moraviensis* BCH24 was found to produce ACC deaminase. In contrast, the evaluation of lytic enzymes revealed that all the tested isolates were positive for urease production, with BCH2 and BCH10 also exhibiting cellulase activity. Additionally, three strains were found to produce protease, amylase, and pectinase (Table 1).

Antagonistic activity

The antifungal activity of five endophytic bacterial strains was evaluated against three phytopathogenic fungi, *Fusarium oxysporum*, *Aspergillus ochraceus*, and *Botrytis cinerea*. *Enterobacter* sp. BCH13 displayed the highest inhibitory effect on *A. ochraceus* (45%), while *Pseudomonas* sp. BCH24 had the greatest antifungal activity against *F. oxysporum* (40%). On the other hand, while *Serratia plymuthica* BCH10 was found to be most effective against *B. cinerea* (35%), *Enterobacter* sp. BCH2 demonstrated efficiency against *F. oxysporum* and *A. ochraceus*, inhibiting their growth by 31% and 27%, respectively (Table 2). These results demonstrate the potential of endophytic bacteria as biocontrol agents against phytopathogenic fungi.

Table 2
Percentage of inhibitions of the endophytic bacteria studied on different phytopathogenic fungi.

	% d'Inhibition		
	Fusarium oxysporum	***Aspergillus ochraceus***	***Botrytis cinerea***
***E. bugandensis* BCH13**	**(26.25 ± 1.44)^{b}**	**(45.83 ± 3.82)^{c}**	-
S. plymuthica **BCH10**	**(16.25 ± 5.20)^{a}**	**(15 ± 7.07)^{a}**	**(35.56 ± 2.22)**
P. moraviensis **BCH24**	**(40 ± 7.64)^{c}**	**(11.25 ± 5.3)^{a}**	-
E. hormaechei **BCH2**	**(31.25 ± 2.50)^{bc}**	**(27.50 ± 4.33)^{b}**	-
P. moraviensis **BCH16**	**(23.75 ± 2.50)^{b}**	**(13.33 ± 7.64)^{a}**	-

Values in the same column followed by the same superscript letter were not significantly different $p < 0.05$ (Fisher's least significant difference (LSD) test).

Halotolerance Assay

The ability of five selected endophytic bacteria to grow in different NaCl concentrations was assessed (Fig. 2). The results showed that the growth rate of the tested strains exhibited an inverse correlation with the NaCl levels, with a decrease in biomass as the salt concentration increased. *Pseudomonas* sp. BCH24 and *Serratia plymuthica* BCH10 were significantly affected by the presence of high levels of NaCl (2.5% – 7.5%), as they demonstrated a great reduction in growth rate. However, *Enterobacter cancerogenus* BCH13 and *Pseudomonas moraviensis* BCH16 were the most salt-sensitive regardless of salinity level (Fig. 2). The adaptability of

the endophytic strains to abiotic stress suggested that BCH13 and BCH16 were salt-tolerant. Subsequently, the two strains were chosen for further testing to determine their ability to promote legume plants under culture chamber conditions.

Endophytic strains enhanced the growth of legumes (Sulla flexuosa **L.)**

A pot trial was conducted to assess the effects of endophytic bacterial strain inoculation on plant growth parameters. After 90 days, all legume plants were harvested to measure growth parameters such as SPAD values, shoot and root heights, as well as fresh and dry weights. Using a SPAD-502 Chlorophyll Meter, the chlorophyll content in the leaves was measured on the day of harvesting. Analysis of chlorophyll content indicated that inoculation of legume plants with a combination of the two endophytes *Enterobacter hormaechei* BCH13 and *Pseudomonas moraviensis* BCH16, along with *R. sullae* KS6, resulted in a considerable increase in chlorophyll content compared to uninoculated treatment and single inoculation (Fig. 3). Under culture chamber conditions, the selected strains were able to exhibit plant growth-promoting properties. Plants inoculated with the rhizobial strain *R. sullae* KS6, BCH13, and BCH16 showed a significant increase in shoot and root height, as well as in fresh and dry weight compared to uninoculated controls. With particular interest, the tri-inoculation test showed the highest significant increase in plant growth compared to the single-inoculation and control treatments, with heights reaching 18.40 ± 1.08 cm and 27.29 ± 5.57 cm respectively for shoots and roots, and values estimated at 10.06 ± 2.84 g and 4.66 ± 0.09g, respectively for fresh shoots and roots weights, as for the dry weights, the values were fixed at 0.76 ± 0.24 g, and 0.31 ± 0.02 cm for shoots and roots (Figs. 4, 5 and 6). These results suggest that tri-inoculation of *Enterobacter hormaechei* BCH13 and *Pseudomonas moraviensis* BCH16 with *R. sullae* KS6 can significantly enhance the growth parameters of *Sulla flexuosa*, even though the origin of the endophytic strains was isolated from another species of legume plants (*Sulla aculeolata* L.).

Discussion

This study aimed to identify endophytic bacterial strains isolated from *Sulla aculeolata* L., a species of legume found in natural the coastal areas of Northeastern Morocco. This research evaluated the potential of phosphate solubilizing endophytes to promote plant growth when cultivated in the field, in accordance with an ecological approach to enhance *Sulla flexuosa* L. growth. Given that the study area is located in a coastal area, plants may be subjected to non-biological stress such as salinity, which can inhibit plant growth and development, particularly in Mediterranean environments (Etesami and Beattie 2017). Therefore, it is imperative to have the ability to withstand high salt concentrations as a crucial criterion when selecting study isolates. halotolerant bacterial endophytes have been identified as promising candidates for use as bio-inoculants to increase salt tolerance of plant growth through maintaining osmotic balance and ion homeostasis (Tufail et al. 2021). In this study, most of the selected strains showed moderate resistance to high-salt environments. The halotolerant phosphate solubilizing bacterial strains BCH13 and BCH16 were identified as *Enterobacter* sp. and *Pseudomonas* sp. and were selected to assess their ability to promote legume plants. The results obtained, highlighted promising PGP attributes of the two strains, with a noticeable increase in the analyzed parameters in comparison with the control groups (Height, dry and fresh weight), similarly, (Enquahone et al. 2022) isolated and characterized 167 halotolerant endophytic bacterial isolates from two Ethiopian soda lakes which showed positive plant growth-promoting properties and tolerance for various abiotic stresses such as salinity, drought, and temperature. Furthermore, Lucero et al. 2021 found that native peanut endophytic phosphate solubilizing bacteria promoted the growth of soybean and maize plants and contributed significantly to their phosphorus content, the strains used in this study also survived in the plant's growth substrate and, in the case of *Enterobacter* sp. J49, successful endophytic colonization in maize and soybean was recorded. Additionally, the selected bacterial isolates have been demonstrated to possess the capacity to produce siderophores and IAA from L-tryptophan. IAA increases root size and distribution, thus enhancing soil nutrient absorption. Siderophores, chelating agents with a strong affinity for ferric irons, facilitate the solubilization of iron, making it available to plant roots in the vicinity. Both mechanisms are considered important properties that significantly improve plant health and growth. Therefore, studies aiming at identifying effective PGP agents, often test for the presence of both compounds. For example, Raja et al. 2019 a investigated 84 endophytic bacteria isolated from nodules and roots of *Cicer arietinum* and *Pisum sativum* plants for siderophore production. Qualitative CAS agar assay revealed 14 isolates produced siderophore and 10 isolates produced siderophore above 65% siderophore units. Additionally, these endophytic bacterial isolates exhibited other plant growth-promoting traits such as IAA, HCN production, and phosphate solubilization. Siderophores can act as biological control agents, limiting the development of plant diseases. Several reports have indicated the positive effects of plant growth-promoting bacteria (PGPB) producing ACC deaminase and IAA on plant growth and stress tolerance. The direct phytostimulatory property of ACC deamination is attributed to the reduction of ethylene levels, which in turn promotes root

elongation (Orozco-Mosqueda et al. 2020). This is consistent with the findings of Kang et al. 2019, who observed that a strain of *Leclercia adecarboxylata* (MO1) that generates ACC deaminase and IAA dramatically improved tomato plant tolerance to salt stress. Of the five isolates tested, only the *Pseudomonas* BCH24 strain was capable of hydrolyzing ACC and producing IAA. On the other hand, the five bacterial endophytes inhibited the growth of at least one fungus. *A. ochraceus* presented the highest sensitivity rate towards *Enterobacter* BCH13. This bio-antagonistic ability could be credited to the production of some hydrolytic enzymes by the selected endophytes besides siderophores and HCN. The extracellular lytic enzymes urease, protease, and cellulase, which are crucial for lysing the pathogenic fungal hyphal wall and competing for nutrients with phytopathogens, can be released by endophytes. Protease, amylase, and pectinase exo-enzymes were equally detected for three endophytes, where only two isolates tested positive for cellulase activity. Egamberdieva et al. 2017 reported the ability of endophytic bacteria to promote plant growth, symbiotic performance, and prevent root rot in chickpeas cultivated in salinated soil. Four bacterial isolates were recovered from chickpea root nodules, including *Bacillus* sp. and *Achromobacter xylosoxidans* NUU2, where *B. subtilis* NUU4 had significant plant growth promotion capabilities, improved rhizobia symbiosis, and reduced the infection rate of root rot caused by *F. solani*. It is a biocontrol strategy in which the control agent consumes nutritional resources before the pathogen, which is advantageous when the fungus grows quickly and covers a large area; nutrient and space competition is one proposed inhibition mechanism for *Aspergillus* species suppression (Lahlali et al. 2022; Dimkić et al. 2023). Comparably, Sharma et al. 2021 studied the diversity pattern and fungicidal activity of bacterial endophytes isolated from two different organic tomato varieties (V1 and V2). A total of 75 bacterial isolates were detected, with *Bacillus* being the most common genus. The data showed that *B. siamensis* (NKIT9) was the most effective antagonist, inducing 75 to 90% of mycelial inhibition against *R. solani*, *B. cinerea*, *F. solani, A. solani*, and *V. lateritium*. The inoculation experiments conducted in this study revealed that tri-inoculation with BCH13 + BCH16 + KS6 resulted in improved legume growth compared to single inoculation and un-inoculated control. The plants inoculated with endophytes and *Rhizobium sullae* exhibited higher levels of chlorophyll, increased root biomass, and as well as shoot biomass. The observed effect is presumably due to the beneficial traits revealed in this study, such as phosphate solubilization, IAA, and siderophores production. The combination of these traits appears to have an additive effect when compared to single inoculation, resulting in improved growth parameters. These findings further substantiate the effectiveness of combined PGPR inoculations in promoting plant growth and productivity and support earlier studies on the synergistic effect of phosphate-solubilizing endophytic bacteria and *Rhizobium* sp. on legume growth (Naveed et al. 2017). For instance, Iličić et al. 2017 reported that the addition of PGP strain *P. chlororaphis* Q16 along with *B. japonicum* strain 526, as compared to the control treatment, improved soybean growth and yield in field conditions. Endophytic bacteria have been shown to improve plant growth, nodulation, and yield through a variety of mechanisms, including the production of IAA, the production of siderophores, and the solubilization of phosphate (Cardoso et al. 2018; Knežević et al. 2021). The interaction of bacteria presenting PGP traits with plants ensures the development and growth of the plants by facilitating nutrient absorption (Elnahal et al. 2022). As an example, results from a study conducted by De vasconcelos martins ferreira et al. 2020 showed that the use of the endophytic rhizobacteria UFLA 02-281/03–18 (*Pseudomonas* sp.), UFLA 02-286 (*Bacillus* sp.), and UFLA 04-227 (*Burkholderia fungorum*) in combination with *Rhizobium tropici* CIAT 899 had a synergistic effect on growth promotion and prevention of damping off in the common bean. These results further validate previous research on the efficacy of some endophytes in biofertilization and biological control of fungal phytopathogens. In this study, growth parameters of Sulla plants (*S. flexuosa* L.) from the Tangier ecotype were improved when inoculated with two endophytes *Enterobacter* sp. and *Pseudomonas* sp.in combination with *R. sullae*, however, no nodulation was observed. This inability is mainly stemming from *R. sullae* since it was obtained from different ecotypes of *S. flexuosa*. It was previously reported that Sulla plants only respond to their specific native species (Thami Alami and El Mzouri 2000; Elyemlahi et al. 2022). However, our findings show that combining the promotion of endophytic plant growth, especially BCH13, and BCH16 with *R. sullae* does indeed have a positive effect on legume productivity, even in the absence of nodules. This could offer several beneficial outcomes such as maximizing legume yields and restoring degraded soils.

Conclusion

The present research has highlighted the presence of endophytic bacteria in *Sulla aculeolata* nodules, the results indicate that the tested strains possess multiple plant growth-promoting properties, in addition to antifungal activity and halotolerant ability. This investigation has equally revealed a synergistic relationship between two endophyte strains, *Enterobacter cancerogenus* BCH13 and *Pseudomonas moraviensis* BCH16, and strain *Rhizobium sullae*, which could be used as ecological bio-inoculants to increase legume production especially legume *Sulla flexuosa*. This research provides a basis for future studies to explore the potential of

endophytic bacteria in association with *Rhizobium* species as a sustainable agricultural practice. Considerably, further investigations are needed to gain a better understanding of the interactions between plant-nodule endophytic bacteria and the effect of the proposed biofertilizer on legume plant growth.

Declarations

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Competing Interests

The authors declare that they have no conflicting interests.

Authors contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Samia HAMANE, Anas EL YEMLAHI and Ouiam ELGALIOU. The first draft of the manuscript was written by Samia HAMANE and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

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Figures

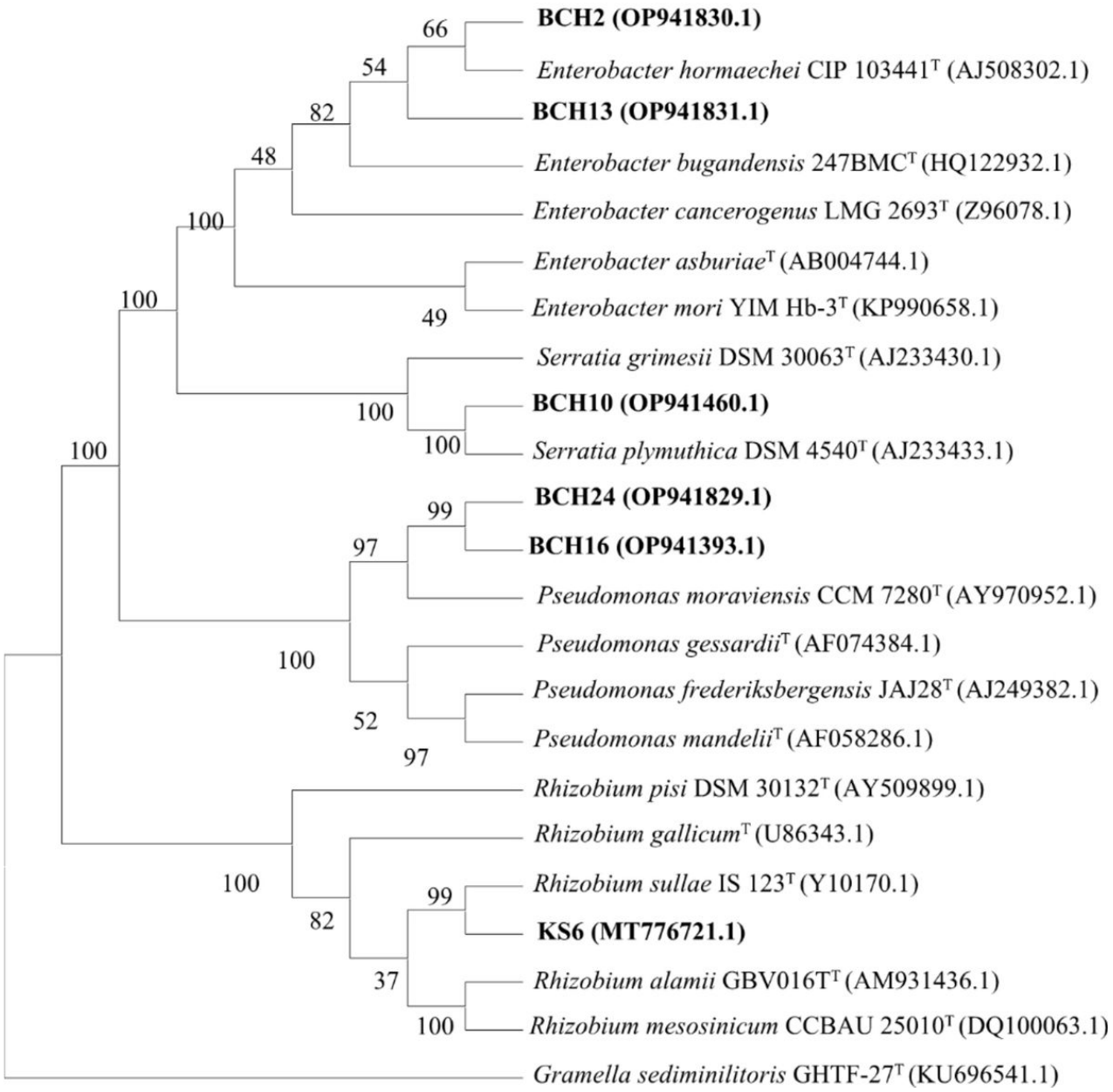

Figure 1

Neighbor-joining phylogenetic tree of 16S rRNA sequences of three isolates (marked with asterisk), from root nodules of Sulla aculeolata L., and phylogenetically related species. The tree is rooted on Gramella sediminilitoris GHTF-27^T.

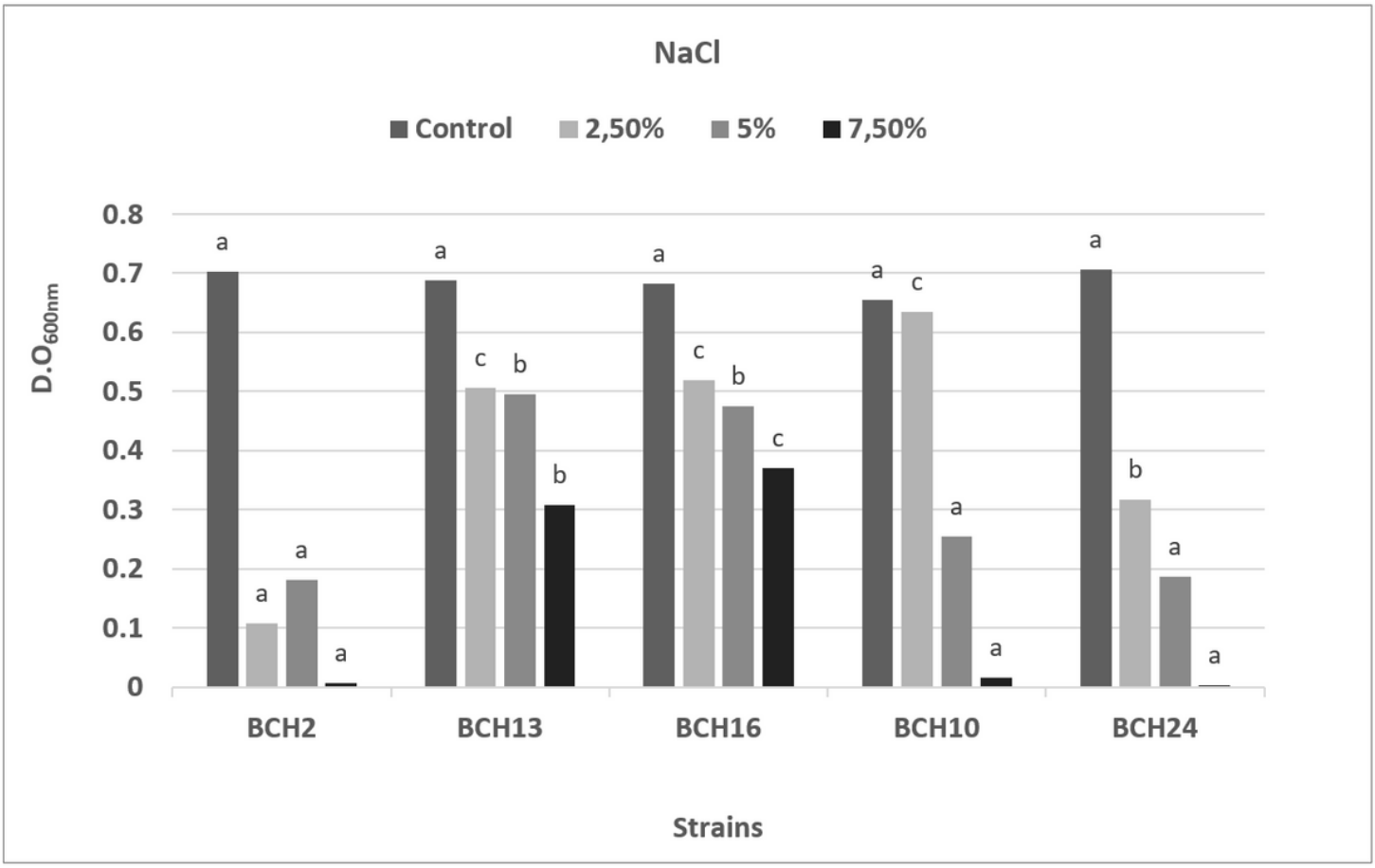

Figure 2

Growth of BSP under non stressed (control) and salinity stressed conditions of different NaCl concentrations. Means in the same column followed by the same letter are not significantly different $p < 0.05$ (Fisher's least significant difference (LSD) test).

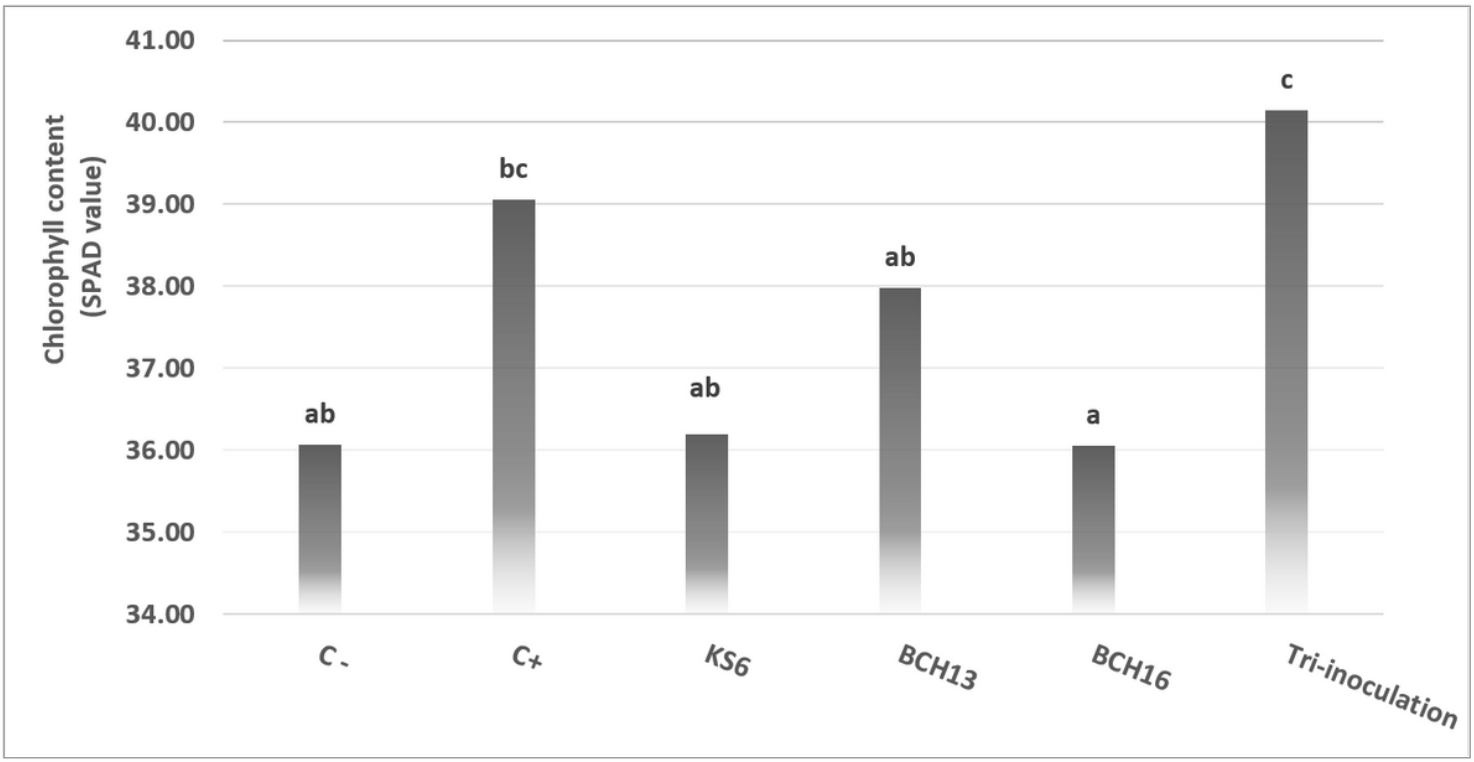

Figure 3

Chlorophyll content in the leaves of inoculated and non-inoculated plants measured by SPAD-502. Means in the same column followed by the same letter are not significantly different $p < 0.05$ (Fisher's least significant difference (LSD) test) (C-, Negative control; C+, Positive control).

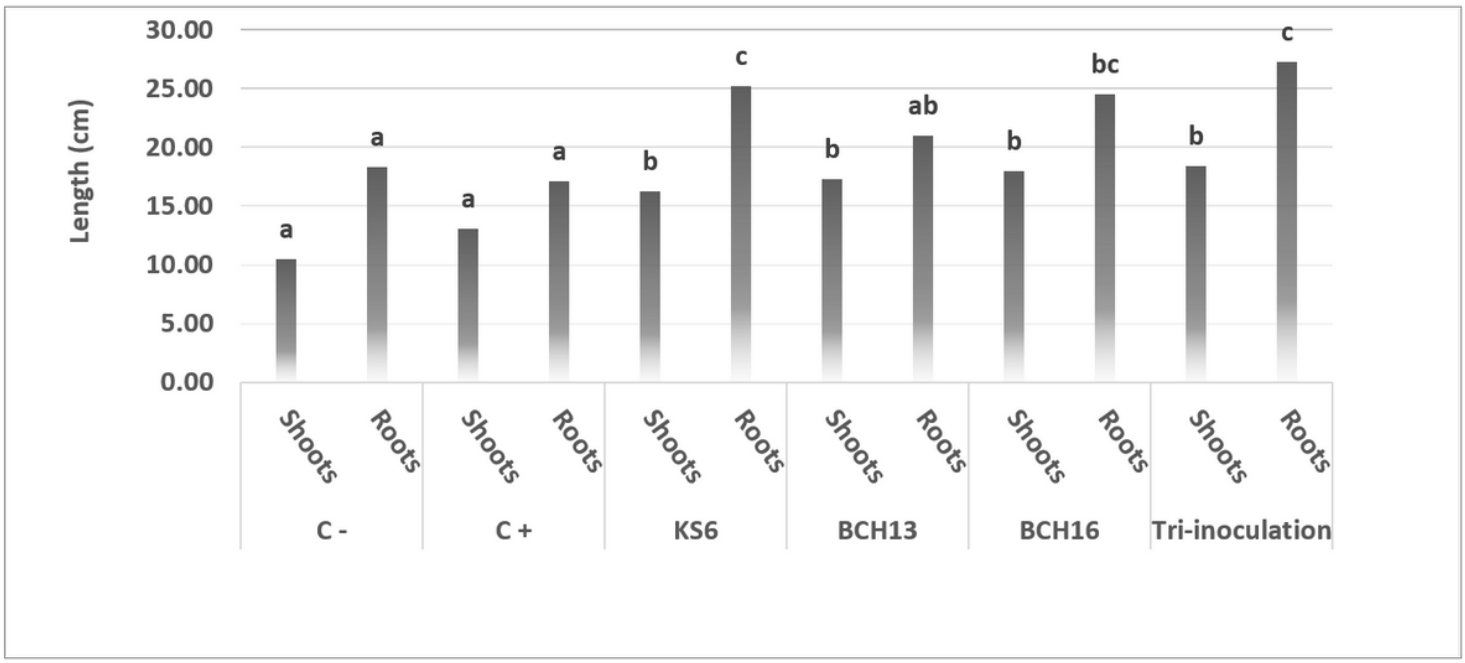

Figure 4

The length of shoots and roots of plants after 90 days of growth in pots. The data presented are the mean of 4 replicates. Means in the same column followed by the same letter are not significantly different $p < 0.05$ (Fisher's least significant difference (LSD) test) (C-, Negative control; C+, Positive control).

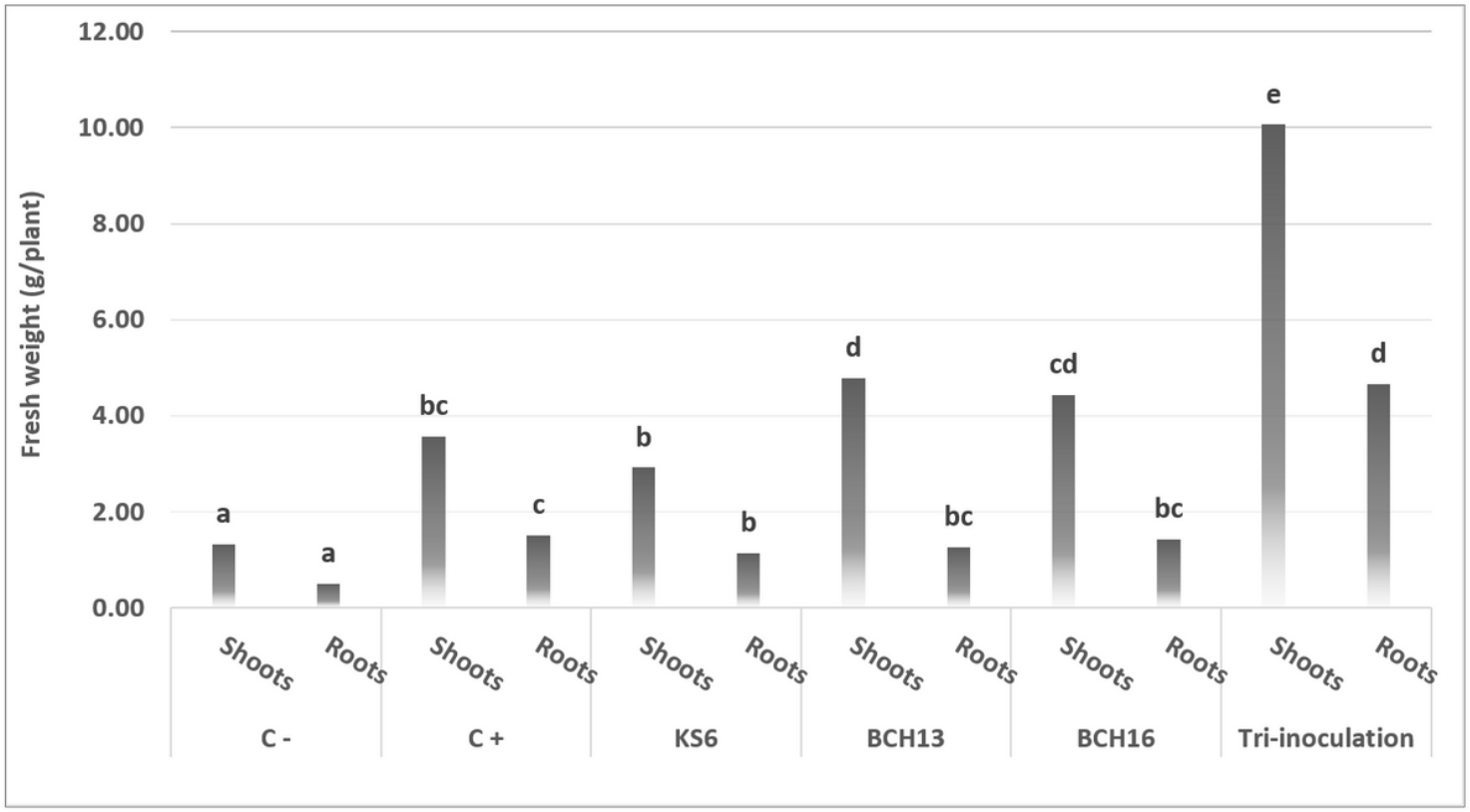

Figure 5

The fresh weight of shoots and roots of plants after 90 days of growth in pots. The data presented are the mean of 4 replicates. Means in the same column followed by the same letter are not significantly different $p < 0.05$ (Fisher's least significant difference (LSD) test) (C-, Negative control; C+, Positive control).

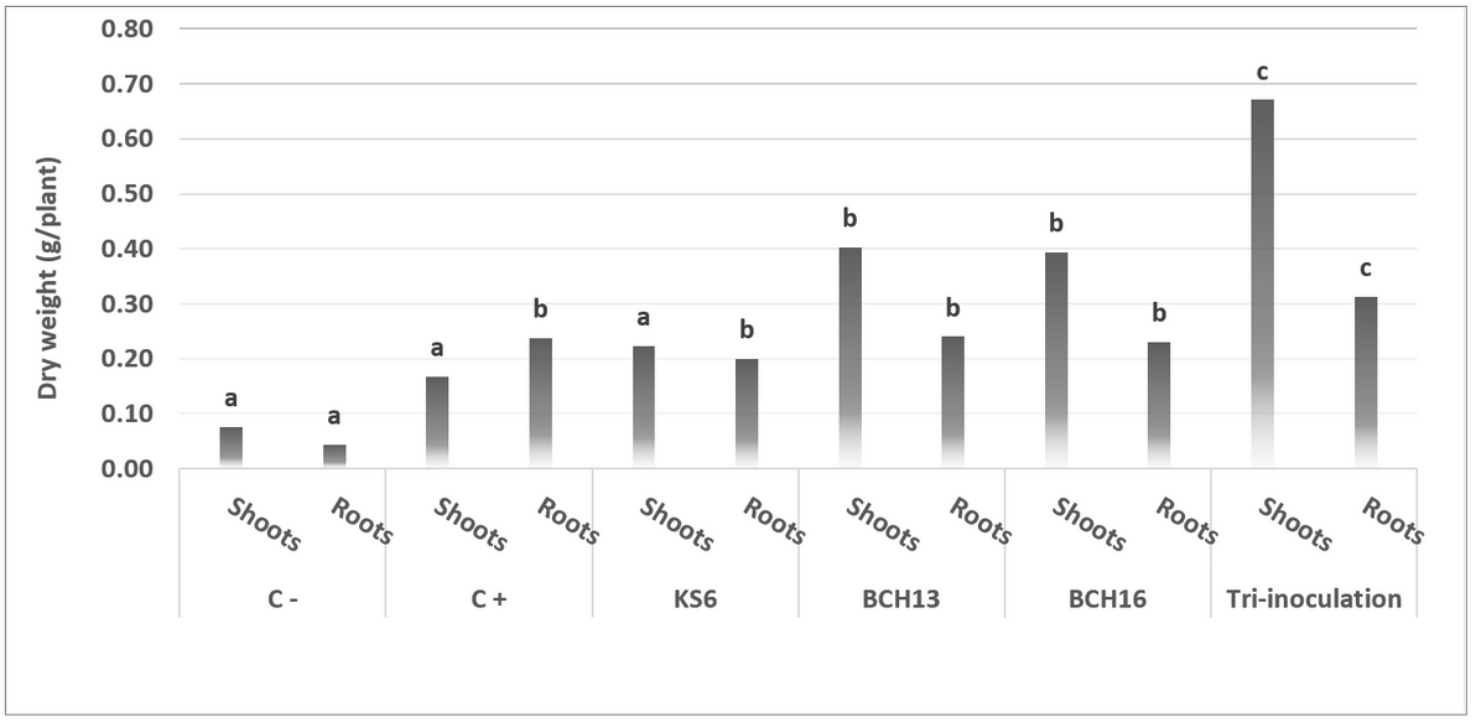

Figure 6

The dry weight of shoots and roots of plants after 90 days of growth in pots. The data presented are the mean of 4 replicates. Means in the same column followed by the same letter are not significantly different $p < 0.05$ (Fisher's least significant difference (LSD) test) (C-, Negative control; C+, Positive control).