

Unraveling the plant growth ~~promotion~~ promoting potential of *Pseudomonas* species isolated from the rhizosphere of *Lotus creticus* ~~grown~~ growing in the Mediterranean coastal regions of Morocco

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Abstract. Rhizosphere-associated bacteria play a key role in enhancing plant performance under saline and nutrient-limited soil conditions, where plant establishment is strongly constrained. In this study, we evaluated the plant growth-promoting potential of rhizobacteria isolated from the rhizosphere of wild-growing *Lotus creticus* ~~growing naturally~~ along the Mediterranean coast of Morocco ~~was evaluated. This is. Of the first report on rhizosphere associated bacteria linked to *L. creticus* in Mediterranean coastal zones. Out of~~ 30 isolates, five bacterial strains, ~~(R125, P79, R8, R150, and R150,)~~ were selected based on their plant growth-promoting ~~rhizobacteria (PGPR)~~ traits. These strains were identified through 16S rRNA gene sequencing as *Pseudomonas protegens*, *Pseudomonas sesami*, *Pseudomonas versuta*, *Pseudomonas helleri*, and *Pseudomonas trivialis*, ~~respectively.~~ Phenotypic characteristics, including IAA production, phosphate solubilization capacity, cellulase and protease activities, and tolerance to salinity and temperature, were evaluated. Additionally, a pot experiment was conducted to assess the impact of inoculation on ~~the growth of *L. creticus*. Significant variation was observed among the selected strains.~~ growth. *Pseudomonas protegens* P79 ~~stood out for its~~ was characterized by strong ~~indole 3-acetic acid (IAA)~~ production, high phosphate solubilization capacity (150.5 mg L^{-1}), and notable cellulase and protease activities. It also demonstrated high tolerance to salinity (up to 13% NaCl) and temperature (up to 45°C). ~~Conversely~~ Comparatively, *Pseudomonas sesami* R8 exhibited broad-spectrum antifungal activity, including strong inhibition of the growth of *Aspergillus ochraceus*. The pot experiment revealed that inoculation with *Pseudomonas helleri* R125 and *Pseudomonas trivialis* R150 significantly ($P < 0.05$) enhanced the aerial dry biomass of *L. creticus* ~~(by 300%), while~~ %. In contrast, *Pseudomonas protegens* P79 was more effective in promoting root elongation ~~(by 8%)~~ % under growth chamber conditions. This study highlights the potential of *Pseudomonas* strains as promising biostimulants for sustainable agriculture and ~~the~~ rehabilitation of coastal marginal soils under combined salinity and nutrient ~~stresses~~ stresses.

Keywords: *Lotus creticus* L., PGPR, *Pseudomonas* spp., biocontrol, 16S rRNA, coastal soils.

1 Introduction

Coastal lands are agroecosystems that support various plant species adapted to saline, ~~and~~ nutrient-poor soils. They also serve as valuable habitats for wildlife, providing food and breeding grounds for livestock (Romano et al., 2022; Younsi and Bouziane, 2023). Despite their ecological importance, these coastal ecosystems are increasingly threatened by climate change, overgrazing, and ~~other~~ anthropogenic pressures, leading to habitat degradation, biodiversity loss, and ~~declining a decline in~~ soil quality (Younsi and Bouziane, 2023). ~~As in~~ Similar to many regions worldwide, Mediterranean coastal soils are generally sandy or weakly developed, exhibiting elevated salinity, low organic matter and nutrient availability, and poor water-holding capacity, which ~~together~~ impose strong constraints on plant establishment and growth (Ferreira et al., 2022; Sun et al., 2026). Therefore, sustainable management and ecological restoration strategies are required to preserve ecosystem ~~functionality~~ function and support agricultural productivity. Legumes (Fabaceae) are widely recognized as effective biological tools for sustainable revegetation and soil restoration (Ainane et al., 2021). ~~Due to~~ Through their ~~capacity~~ ability to fix atmospheric nitrogen in symbiosis with ~~rhizobial~~ soil bacteria, ~~legumes enhance~~ these plants contribute to soil fertility, thereby reducing the need for ~~chemical~~ synthetic fertilizers and ~~promoting~~ supporting sustainable ~~farming~~ agricultural systems (Abd-Alla et al., 2023). ~~Among them the diverse legume species found in Mediterranean ecosystems,~~ *Lotus creticus* L., ~~is a~~ perennial ~~legume native to the Mediterranean region, is~~ species particularly well adapted to saline and arid coastal environments (Belechheb et al., 2021). Its high ~~tolerance to~~ salinity and ~~its ability to thrive under~~ poor soil ~~tolerance~~ conditions make it a promising candidate for soil stabilization and rehabilitation of degraded coastal areas (Navarro et al., 2023). Like many leguminous plants, *L. creticus* may harbor beneficial bacteria known as plant growth-promoting rhizobacteria (PGPR) in its rhizosphere. These bacteria have been extensively studied and ~~have~~ demonstrated significant potential to enhance plant growth and stress tolerance under saline and nutrient-limited conditions (Vejan et al., 2016; de Andrade et al., 2023; Nisa et al., 2026). They promote plant performance through multiple mechanisms, including nutrient acquisition, phytohormone production, and stress alleviation (Sharma et al., 2025). For instance, phosphate solubilization improves phosphorus availability in calcareous soils, siderophore production enhances iron uptake under alkaline conditions, and ACC deaminase activity reduces ethylene-induced stress under ~~salinity~~ saline conditions (Pattnaik et al., 2021; Khan et al., 2022; Yang et al., 2026). Additionally, these microorganisms can induce systemic tolerance and improve plant resilience to abiotic stresses such as drought and salinity (Bashan et al., 2014; Mekureyaw et al., 2022; Salwan et al., 2023). Although most investigations have ~~centered~~ focused on cultivated species ~~like~~ such as wheat, rice, and maize, the rhizobacterial communities of wild coastal legumes, particularly *Lotus creticus*, remain ~~insufficiently explored under~~ explored. Under coastal environmental conditions, rhizosphere processes, particularly ~~microbial~~ microbe-mediated nutrient mobilization, play a key role in plant survival and growth (Adeleke et al., 2024; Chu et al., 2025). ~~These~~ These environments also represent environmentally challenging ecosystems that provide a relevant natural model for studying plant-microbe interactions under combined salinity and nutrient limitations (Marien et al., 2023).

In this context, assessing the functional diversity of rhizobacteria associated with wild coastal legumes is ~~key to~~important for understanding how soil limitations shape microbially mediated plant adaptation ~~into~~ saline coastal ~~soils~~soil. Therefore, this study ~~aims~~aimed to isolate and characterize rhizobacteria associated with *L. creticus*, evaluate their plant growth-promoting traits, and assess their potential as bioinoculants ~~to improve~~for improving plant performance in Mediterranean coastal environments. ~~Such~~These results provide new insights into the role of rhizosphere bacteria in enhancing plant growth and adaptation ~~under~~to saline and nutrient-limited conditions.

2 Materials and methods

2.1 Plant and soil sampling

Plant samples of *L. creticus* were collected in March 2024 from a natural population of *L. creticus* growing wild in the coastal region of northwestern Morocco, near Tahadart Beach (35°34'56.0"N, 5°59'23.5"W). The region is characterized by a Mediterranean climate, with approximately 525 mm of annual precipitation and an average temperature of 19.1 °C. ~~Sampling was conducted at~~Climatic data for the sampling site, including annual precipitation and seasonal temperatures, were obtained from the National Oceanic and Atmospheric Administration (NOAA) National Centers for Environmental Information (NCEI) database, available at <https://www.ncei.noaa.gov>. ~~Sampling was conducted during~~ the late flowering stage on mobile dunes. Three independent biological replicate plots of 1 m² were randomly established ~~from~~in the area near the seashore to capture spatial variability. Each replicate ~~corresponds~~corresponded to a composite sample collected from each plot. ~~Samples~~The samples (stems and leaves) were oven-dried at 60 °C to constant weight prior to analysis. The dried samples were analyzed for crude protein content using the Kjeldahl method (AOAC, 1997) and ~~for~~organic matter by incineration at 550 °C for 12 h (AOAC, 1997).

~~For bacterial isolation, plant~~Plant roots from each plot were carefully excavated, and loosely adhering soil was removed by gentle shaking and collected in sterile bags. ~~The soil~~Soil tightly attached to the roots was ~~then~~recovered by brushing the roots into the same bags. ~~Also~~In addition, rhizosphere soil firmly adhering to the roots was sampled from the 0-20 cm surface layer. Electrical conductivity (EC) ~~is~~was measured ~~by using~~ using the saturated paste method ~~using with~~ an ORION brand conductivity meter, model 162 (USSLS, 1954). The exchangeable sodium percentage (ESP) was calculated as $ESP = (Na^+ / CEC) \times 100$, where CEC represents the cation exchange capacity (FAO, 1970). Total soil nitrogen was measured using the Kjeldahl method (ISO, 1995). ~~Total~~Available phosphorus was assessed using the Olsen method (Olsen et al., 1954), and exchangeable potassium was measured ~~by using~~ flame photometry, following the method of Bower et al. (1952). Soil organic matter was evaluated using ~~the~~Walkley and ~~Black~~Black's method (Walkley and Black, 1934).

2.2 Isolation of rhizobacteria

One gram of ~~rhizospheric~~rhizosphere soil was mixed with 9 mL of sterile NaCl solution (0.9% w/v). The mixture was stirred at 200 rpm for 1 ~~hour~~h and subjected to serial dilutions ranging from 10^{-1} to 10^{-7} . Then, 100 µL ~~from~~of each dilution was plated onto Petri dishes containing King B (KB) medium and incubated at 28 °C for 3 days. After incubation, single colonies were selected and repeatedly streaked onto KB agar medium until pure colonies were obtained. Finally, pure isolates were preserved in 25% (v/v) sterile glycerol at ~~-~~20 °C until analysis.

2.3 Morphological characterization and Gram staining

The morphological characteristics of each colony, including shape, structure, and pigmentation, were examined after streaking the bacteria ~~were streaked~~ onto KB agar plates. ~~Additionally~~, Gram staining was performed on fixed smears of bacterial cultures following the method described by Cyrabree and Hindshill (1975).

2.4 Molecular characterization

Bacterial genomic DNA was extracted using the phenol-chloroform method described by Chen and Kuo (1993). DNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop 2000). The nearly full-length 16S rRNA gene was amplified using the universal primers rD1 and fD1, as described by Weisburg et al. (1991). PCR products were verified by horizontal agarose gel electrophoresis (70 V for 1 h) in Tris-acetate-EDTA (TAE) buffer. The amplified fragments were purified and subjected to cycle sequencing using the same primer ~~sets~~sets. The obtained sequences (≈1500 bp) were assembled and edited using BioEdit software (version 7.0.5.3) and manually checked for quality. Sequence similarity searches were performed using the BLAST algorithm against the GenBank database. The closest type strains and their corresponding sequence similarities (%) were recorded. The nucleotide sequences generated in this study were deposited in GenBank under accession numbers: OM403655, OM420233, OQ324690, OM317560, and OM403658. For phylogenetic analysis, sequences were aligned with reference sequences retrieved from GenBank using ClustalW implemented in MEGA version 11. A phylogenetic tree was constructed using the neighbor-joining method based on Kimura's two-parameter model. The robustness of the tree topology was evaluated by using bootstrap analysis with 1000 replicates.

2.5 Assessment of PGP traits

2.5.1 Phosphate solubilization

~~The bacterial~~Bacterial isolates were tested for their ability to solubilize tricalcium phosphate (TCP), which increases the pool of plant-available phosphorus in ~~soil~~the soil. A 10 µL aliquot of fresh bacterial culture was inoculated onto PVK agar medium, which served as the sole phosphorus source, and incubated at 28 °C for ~~7~~seven days (Pikovskaya, 1948). The plates were checked for halo formation, and the solubilization index (SI) was calculated using the formula described by Ed-Premono et al. (1996):

$$SI = \frac{\text{Colony diameter} + \text{Halo diameter}}{\text{Colony diameter}}$$

The isolates were further evaluated for their ability to solubilize tricalcium phosphate (TCP) in liquid medium by inoculating 500 µL of bacterial culture into 50 mL ~~of~~ PVK liquid medium. Sterile, uninoculated media ~~served~~were used as controls. Both inoculated and control media were incubated for 7 days at 28 °C on an orbital shaker at 180 rpm. After incubation, the cultures

were centrifuged at 13,000 rpm for 20 ~~minutes~~min. The concentration of soluble phosphorus in the supernatant was determined using the colorimetric method ~~developed~~described by Ames (1966).

2.5.2 Production of ammonia

Through nitrogen enrichment and biocontrol in the rhizosphere, ammonia production improves nutrient availability and contributes to plant growth under ~~stress~~stressful conditions (Yang et al., 2025). Ammonia production was assessed ~~following the method~~as described by Cappuccino and Sherman (1992). Bacterial cultures were inoculated into 10 mL of peptone water and incubated at 36 ± 2 °C for 48 ~~to~~72 ~~hours~~h. Ammonia production was indicated by a color change from yellow to brown ~~upon~~after the addition of Nessler's reagent.

2.5.3 Production of hydrogen cyanide (HCN)

The isolates were evaluated for their ability to produce hydrogen cyanide (HCN), a key trait involved in ~~the suppression of~~suppressing soil-borne pathogens and ~~the maintenance of~~maintaining rhizosphere health (Sehrawat et al., 2022). To estimate HCN production, 100 µL of bacterial culture was streaked onto KB agar plates supplemented with 4.4 g of glycine. Filter paper ~~disks~~disks (9 cm in diameter), saturated with 2% sodium carbonate, were then placed inside each Petri plate. The plates were sealed with ~~parafilm~~Parafilm and incubated at 28 °C. A color change from yellow to orange or brown ~~indicated~~indicates HCN production (Bakker and Schippers, 1987).

2.5.4 Production of siderophores

Siderophore secretion facilitates iron acquisition under limiting conditions by chelating Fe^{3+} and improving its bioavailability ~~to~~in plants (Timofeeva et al., 2022). The bacterial isolates were spot-inoculated onto KB agar medium to assess siderophore production and incubated for 3 ~~days~~d at 28 °C. Subsequently, a layer of Chrome Azurol S (CAS) medium was overlaid ~~on~~on the surface of the plates, which were then incubated in the dark for 24 ~~hours~~h. A color change in the CAS medium from blue to orange ~~indicated~~indicates siderophore production (Schwyn and Neilands, 1987).

2.5.5 Production of ACC deaminase

ACC (1-aminocyclopropane-1-carboxylate) deaminase activity enables rhizobacteria to mitigate plant stress by lowering ethylene levels through the degradation of its ~~precursor~~precursors (Orozco-Mosqueda et al., 2020). The isolates were evaluated for their ability to synthesize ACC deaminase, using ACC as the sole nitrogen source, following the procedure described by Jacobson et al. (1994). Bacterial growth was assessed by measuring ~~the~~ optical density (OD) at 600 nm. Higher OD values indicated increased ACC deaminase activity. ~~Results~~The results were compared to those obtained using a control medium containing an alternative nitrogen source, such as ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$).

2.5.6 Production of IAA

IAA (~~Indole~~indole-3-acetic acid) is a phytohormone involved in the regulation of root development and ~~the~~ stimulation of cell elongation and division (Etesami and Glick, 2024). To evaluate the ability of the isolated bacteria to synthesize IAA, 0.05% tryptophan was added to ~~the~~ TSA medium. After solidification, a sterile nitrocellulose membrane was carefully placed on the

160 surface of the medium and inoculated with 10 µL of bacterial culture. Following incubation at 28 °C, the membrane containing the colonies was transferred onto ~~a~~-Whatman filter paper impregnated with Salkowski reagent (2% FeCl₃ [0.5 M] in 35% perchloric acid). Bacteria capable of synthesizing IAA were identified by the appearance of red halos surrounding ~~the~~their colonies (Bric et al., 1991).

2.5.7 Production of extracellular enzymes

165 ~~Production of cellulase. Through the degradation of cellulose rich compounds, cellulase~~Cellulase production. Cellulase production supports carbon transformation and nutrient recycling in soil environments through the degradation of cellulose-rich compounds (Datta et al., 2024). The strains were cultured on ~~CMC~~-(carboxymethyl cellulose (CMC)) agar plates. The ~~medium's pH of the medium~~ was adjusted to 7, and the plates were incubated at 30 °C for 5 days. After incubation, the plates were soaked in an aqueous solution of Congo red (0.1%) and ~~then~~-rinsed with 1 M NaCl solution. The formation of a clear zone around the colonies indicated cellulose degradation (Miller, 1959).

Production of amylase. Amylase activity reflects the capacity of microorganisms to hydrolyze starch into assimilable sugars, supporting microbial metabolism and soil biochemical processes (Morbia et al., 2024). ~~For the quantification of~~To assess amylase production, 10 µL of fresh bacterial culture was inoculated onto starch agar medium (SAM) and incubated at 37 °C for 48 ~~hoursh~~. The plates were then soaked in ~~a~~-1% iodine solution for 5 ~~minutes~~min. The appearance of a clear zone around 175 the bacterial colonies indicated amylase production (Collins et al., 2004).

~~Production of chitinase. Chitinase production.~~ Chitinase is ~~linked to~~associated with antifungal activity through the enzymatic degradation of chitin, a major structural component of fungal cell walls (Veliz et al., 2017). To confirm chitinase synthesis by the bacterial isolates, colloidal chitin (C₈H₁₃O₅N)_n was used as ~~thea~~ carbon source (Renwick et al., 1991). Petri dishes containing colloidal chitin agar medium were inoculated with freshly prepared bacterial ~~cultures~~culture. After 180 incubation at 30 °C for 4 days, positive colonies were surrounded by distinct, clear halos, indicating chitin degradation.

~~Production of protease. Protease production.~~ Protease activity contributes to nitrogen cycling by facilitating the degradation of proteinaceous substrates and may also play a role in suppressing pathogenic microorganisms (Adameczyk et al., 2021). Protease production ~~iswas~~ assessed using a skim milk agar medium prepared as described by Vijayaraghavan and Vincent (2013). The plates were inoculated with 100 µL of fresh bacterial culture and incubated at 28°C for 48 ~~hoursh~~. The appearance 185 of a clear halo around the ~~eolony indicates~~colonies indicated protein degradation.

~~Production of urease. Urease production.~~ Urease activity catalyzes the hydrolysis of urea into ammonia, contributing to nitrogen transformation in ~~soilssoil~~ (Zhang et al., 2026). Urease production was detected by the alkalization of Christensen's urea agar medium, which contained urea as the sole nitrogen source and phenol red as ~~thea~~ pH indicator. ~~Plates~~The plates were inoculated with the bacterial strains and incubated at 30 °C for 48 ~~hoursh~~. After incubation, positive colonies were identified 190 by ~~athair~~ pink-purple coloration against a yellow background (Brink, 2010).

~~Production of catalase. Catalase production.~~ By converting hydrogen peroxide into water and oxygen, catalase plays a key role in protecting cells from oxidative damage caused by reactive ~~oxygen~~-species (Roy et al., 2022). The catalase test was

performed by adding a drop of 3% hydrogen peroxide ~~ontoto~~ a freshly cultured bacterial isolate on a sterile slide using a sterile inoculation loop. The appearance of effervescence indicated catalase activity (Bumunang and Babalola, 2014).

2.6 Antagonism against phytopathogenic fungi

The antagonistic potential of rhizobacteria against phytopathogens ~~representsis~~ a critical mechanism of biological control, limiting pathogen growth through direct or indirect interactions (Espinosa-Palomeque et al., 2025). The bacteria were assessed for their potential to inhibit the growth of four plant pathogenic fungi, *Fusarium oxysporum*, *Botrytis cinerea*, *Aspergillus ochraceus*, and *Aspergillus flavus*, chosen for their prevalence and significant impact on various important legumes in the Mediterranean climate, including *L. creticus* (Maciá-Vicente et al., 2008; Tsitsigiannis et al., 2012); ~~El Aaraj et al., 2015).~~ Antifungal activity was evaluated using potato dextrose agar (PDA) medium, as described by Rabindran and Vidhyasekaran (1996). ~~The bacteria were assessed for their potential to inhibit the growth of plant pathogenic fungi, including Fusarium oxysporum, Botrytis cinerea, Aspergillus ochraceus, and Aspergillus flavus, which were isolated and characterized by El Aaraj et al. (2015).~~ A 5 mm agar disk from a fresh fungal culture was placed at the center of Petri plates filled with PDA medium. Subsequently, 20 µL of each bacterial culture was inoculated as spots 3 cm away from the fungal strain. Control experiments were conducted without bacteria. The plates were incubated at 25 °C for 7 days and examined for fungal inhibition using the following formula:

$$\% \text{ Inhibition of fungal growth} = 100 \times \frac{(r_1 - r_2)}{r_1}$$

~~With ‘‘r1’’~~where r_1 is the diameter of the control culture, and ~~‘‘r2’’~~ r_2 is the diameter of the treated culture.

2.7 Stress effect

2.7.1 Effect of NaCl

~~Salt~~The salt tolerance reflects the ability of microorganisms to survive and maintain metabolic activity under high osmotic pressure conditions typical of saline environments (Witzel et al., 2025). Salt tolerance of the selected ~~strains~~strains was evaluated by streaking a single colony onto Petri dishes containing King’s B (KB) medium supplemented with increasing NaCl concentrations ranging from 0 ~~to~~ 14% (w/v). ~~Plates~~The plates were incubated at 28 °C for 48 h in the dark. Bacterial growth was ~~assessed~~qualitatively assessed based on colony formation and compared with control plates without NaCl. All assays were performed in triplicate (Novitsky and Kushner, 1975).

2.7.2 Effect of Temperature

Temperature tolerance indicates the capacity of bacterial strains to withstand thermal stress and maintain functional stability ~~aerossunder~~ a range of environmental conditions (Sharma et al., 2025). The tolerance of the bacterial isolates to heat stress was assessed by streaking them onto King’s B (KB) agar plates and incubating at various temperatures (30, 37, 42, 45, and 50 °C) for 24 h in the dark. Growth was evaluated based on colony formation and compared to control plates incubated at 28 °C. All experiments were ~~conducted~~performed in triplicate.

2.7.3 Effect of pH

The ability to tolerate pH variations determines microbial viability and function by modulating enzyme activity and cellular homeostasis in acidic ~~or~~and alkaline soils (Msimbira and Smith, 2020). Tolerance of the isolates to acidic and alkaline conditions was evaluated by streaking ~~bacterial isolates~~them onto buffered King’s B (KB) medium adjusted to pH values of 3, 5, 6, 6.5, 7.5, 8.5, 9, 10, and ~~10~~11. Bacterial growth reflected tolerance to the tested pH, ~~while~~whereas its absence indicated sensitivity.

2.8 Inoculation of *Lotus creticus*

The seeds of *L. creticus* were disinfected by immersion in 95% ethanol for 1 ~~minutemin~~, followed by treatment with 1.2% sodium hypochlorite for 20 ~~minutesmin~~, and then thoroughly rinsed several times with sterile water. The seeds were placed on filter paper ~~disesdisks~~ moistened with ~~10 mL~~10 mL of sterile water in a Petri dish and incubated at ~~28~~28 °C. Three germinated seeds were then planted in plastic pots (25 × 22 × 15 cm) filled with 1 kg of sterilized soil obtained from the same location where *L. creticus* plants were originally collected and directly inoculated with 1 mL per seed of bacterial culture (10⁸ colony-forming units/mL) grown in TSB. Each treatment consisted of three pots. ~~Uninoculated~~The uninoculated pots served as controls. All pots were maintained under greenhouse conditions ~~maintained~~at 25 °C~~under~~, with a 16:8 h light-dark photoperiod, and irrigated weekly with distilled water~~and/or a~~. Additionally, the mineral solution ~~(described by Broughton and Dilworth, 1970)– (1971), containing 500 μM KH₂PO₄, 1000 μM CaCl₂·2H₂O, 250 μM MgSO₄·7H₂O, 250 μM K₂SO₄, 10 μM ferric citrate, 1 μM MnSO₄·H₂O, 2 μM H₃BO₃, 0.5 μM ZnSO₄·7H₂O, 0.2 μM CuSO₄·5H₂O, 0.1 μM CoSO₄·7H₂O, and 0.1 μM Na₂MoO₄·2H₂O and adjusted to pH 6.8, was applied every three months throughout the experiment.~~

2.9 Statistical analysis

All experiments were performed in three independent replicates and presented as means ± standard deviation (SD). Differences ~~amongbetween treatment~~ means were ~~evaluateddetermined~~ using analysis of variance (ANOVA)~~onee~~), following verification of normality and homogeneity of variances~~had been verified~~. Significant differences were assessed using Fisher’s Protected Least Significant Difference (LSD) test ($P < 0.05$). Statistical analyses were conducted using STATISTICA 13.3 software.

3 Results

3.1 Soil analysis and symbiosis evaluation

The results of the soil analysis (Table 1) ~~indicated the occurrence of~~indicate that *L. creticus* occurs in a sandy Arenosol, characterized by a high proportion of sand and low levels of clay and silt~~fractions~~. The soil was alkaline (pH= 8.4), ~~showedexhibited~~ moderate salinity (EC = 4.2 mS/cm), and ~~had a~~low exchangeable sodium percentage (ESP = 6.3%). It was also nutrient-poor, ~~containingwith~~ very low organic matter content (0.48%) and limited ~~amountsconcentrations~~ of available phosphorus (3 ppm), potassium (45.2 ppm), and nitrogen (0.08%).

Table 1. Soil physicochemical properties of the sampling site

WRB Class	C (%)	FSi (%)	CSi (%)	FS (%)	CS (%)	EC (mS/cm)	pH (H ₂ O)	pH (KCl 1N)	OM (%)	P ₂ O ₅ (ppm)	K ₂ O (ppm)	N (%)	ESP (%)
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Arenosol	1.330 ± 0.30	1.330 ± 0.10	3.660 ± 0.10	69.5 ± 0.50	24.33 0± 0.20	4.220 ± 0.10	8.4 ± 0.10	9.110 ± 0.50	0.484 0 ± 0.40	3.00 ± 0.20	45.220 ± 0.10	0.08 ± 0.10	6.330 ± 0.50
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WRB: World Reference Base, C: Clay, FSi: Fine Silt, CSi: Coarse Silt, FS: Fine Sand, CS: Coarse Sand, EC: Electrical conductivity, OM: Organic matter, ESP: exchangeable Sodium Percentage. Values are expressed as the mean ± SD of three replicates.

Additionally, plant examination ~~showed~~revealed a strong ~~symbiotic ability of nodulation capacity in~~ *L. creticus*, ~~forming as evidenced by the formation of~~ large, pink nodules ~~as assessed using the nodule scoring chart proposed by Howieson and Dilworth (2016) (Table 2).~~ The results ~~(Table 2)~~ demonstrated the high productivity of *L. creticus*, ~~yielding which yielded~~ up to 781.25 kg of dry matter per hectare. The plant ~~produced~~exhibited high organic matter (91.32% DM) and crude protein (12.60% DM) ~~content at contents during~~ the flowering stage.

Table 2. Nodulation and growth of *L. creticus*

Nodulation ¹	DM (Kg/ha)	OM (%DM)	CP (%DM)
Abundant	781.25 ± 08.75	91.32 ± 0.38	12.60 ± 0.56

~~Values are presented as the mean of 3 replicates.~~ DM: dry matter, OM: organic matter, CP: crude protein. ¹: using ~~at the~~ nodule-scoring chart proposed by Howieson and Dilworth (2016). Values are expressed as the mean ± SD of three replicates.

3.2 Isolation and morphological characterization of rhizobacteria

Thirty bacteria were isolated from the rhizosphere of distinct *L. creticus* plants and cultured in King B medium. Five isolates were selected for further examination based on their growth characteristics. Morphologically, three isolates (~~P8, P15R8, R15,~~ and P79) exhibited ~~bacillus rod-shaped colonies, while cells (bacilli), whereas~~ two isolates (~~P115R150 and P125R125~~) displayed ~~spherical cells (cocci-shaped colonies).~~ All isolates were identified as Gram-negative bacteria and formed cream-colored colonies, except for P79, which produced green ~~colored~~ colonies.

3.3 Molecular characterization

Electrophoretic analysis ~~showed~~revealed distinct amplicons of ~~about~~approximately 1500 bp in all bacterial isolates, corresponding to the expected size of the 16S rRNA gene (Figure 1).

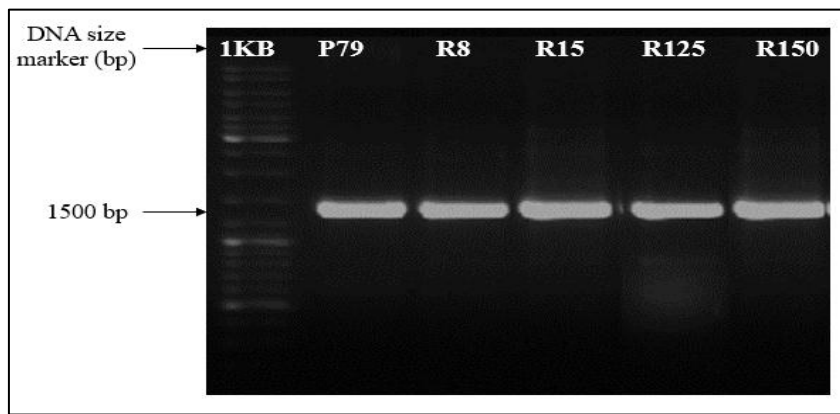


Figure 1: Agarose gel electrophoresis analysis of 16S rRNA genes amplified from 5 bacterial isolates.

285 ~~The genetic~~Genetic characterization of the selected strains revealed a close phylogenetic relationship to the *Pseudomonas* genus based on nearly complete 16S rRNA gene sequences, with similarity values exceeding 98%. According to the phylogenetic tree (Figure 2) inferred from the 16S rRNA sequences, the five strains P79, R8, R15, R125, and R150 isolated from the ~~rhizospheric~~rhizosphere soil of *L. creticus* were closely related to *Pseudomonas protegens*, *Pseudomonas sesami*, *Pseudomonas versuta*, *Pseudomonas helleri*, and *Pseudomonas trivialis*, ~~respectively.~~ The 16S rRNA gene sequences of these

290 five *Pseudomonas* strains ~~have been~~were submitted to NCBI GenBank, and their accession numbers are provided in Figure 2.

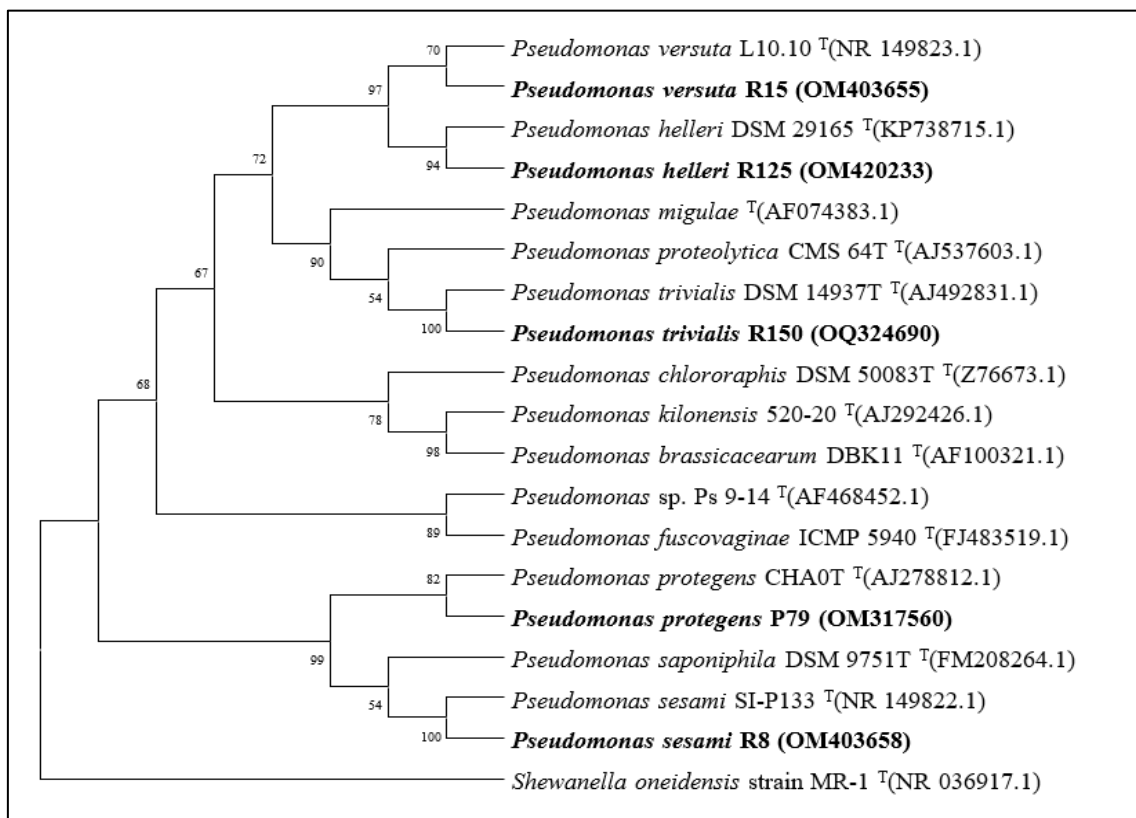


Figure 2: Phylogenetic tree based on the 16S rRNA gene sequences showing the relationship ~~of between~~ the five *Pseudomonas* strains (highlighted in the tree) and other related species. The tree was reconstructed ~~by using~~ the ~~Neighbor-Joining~~ neighbor-joining tree method. Bootstrap values are indicated as percentages derived from 1000 ~~replications~~ replicates. The tree ~~is was~~ rooted with *Shewanella oneidensis* strain MR-1^T

3.4 Assessment of PGP traits

The plant growth-promoting properties of the five selected bacterial strains are ~~summarized~~ presented in Table 3. The results revealed ~~significant variation~~ marked differences in their ability to solubilize phosphate and acidify the culture medium.

Table 3. Plant growth-promoting traits of the five selected rhizobacterial strains.

Strain	S. I	P solubilization	pH	HCN	Siderophores	Ammonia	IAA	ACCD
P79	1.70 ± 0.440	150.50 ± 0.220	5.16 ± 0.660	++	+	++	+++	+
R8	1.50 ± 0.410	59.70 ± 0.550	6.11 ± 0.440	+++	++	+++	+	+
R15	1.70 ± 0.550	76.09 ± 0.330	6.16 ± 0.410	+	-	++	+	+

R125	1.40 ± 0.220	91.25 ± 0.330	5.17 ± 0.550	-	++	+++	+	+
R150	1.40 ± 0.330	87.05 ± 0.410	4.65 ± 0.220	++	++	+++	+	+

Values are presented as the ~~mean~~means of ~~3~~three replicates. S.I: solubilization index; IAA: Indole acetic acid; ACCD: ACC deaminase; P solubilization in mg·L⁻¹; +: low activity; ++, ++: moderate activity; +++, +++: strong activity; -, -: no activity.

Pseudomonas protegens P79 ~~exhibited~~demonstrated the highest phosphate solubilization (150.5 mg·L⁻¹) ~~along with and~~ moderate siderophore production. *Pseudomonas sesami* R8 ~~stood out was notable~~ for its strong HCN production. However, it displayed the lowest phosphate solubilization capacity (59.70 mg·L⁻¹). *Pseudomonas helleri* R125 and *Pseudomonas trivialis* R150 ~~showed~~exhibited similar profiles, both exhibiting moderate phosphate solubilization (91.25 and 87.05 mg·L⁻¹, respectively) and ammonia production. *Pseudomonas versuta* R15 ~~demonstrated~~showed relatively low phosphate solubilization (76.09 mg·L⁻¹) and weak HCN production, and ~~it was unable to~~could not produce siderophores. All strains ~~produced~~exhibited low IAA levels and ACC deaminase, ~~though only at low levels activity, except for P79, which exhibited high IAA production.~~

3.5 3.5 Production of extracellular enzymes

Table 4 highlights the ability of the isolates to produce extracellular enzymes, including cellulase, chitinase, amylase, protease, urease, and catalase. All bacterial strains exhibited minimal enzymatic activity, except ~~for~~ *Pseudomonas protegens* P79, which ~~showed significant~~exhibited high cellulase and protease activities. This strain ~~also~~exhibited moderate chitinase production, ~~along with and~~ low ~~levels of~~urease and catalase activity.

Table 4. Extracellular ~~enzymes~~enzyme production by ~~the~~bacterial isolates.

Strain	Cellulase	Chitinase	Amylase	Protease	Urease	Catalase
P79	+++	++	++	+++	+	+
R8	+	-	+	-	++	+
R15	+	+	+	+	++	+
R125	+	+	+	-	+	+
R150	+	+	+	+	-	+

(+): low activity, (++): moderate activity, (+++): strong activity, (-): no activity. Values are presented as the ~~mean~~means of ~~3~~three replicates.

3.6 Antagonism against phytopathogenic fungi

The antagonistic activity of the five rhizobacterial strains against the four fungal pathogens, *Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus ochraceus*, and *Botrytis cinerea*, is presented,shown in Figure 3. Notably, *Pseudomonas sesami* R8 exhibited a broad-spectrum-of antifungal activity compared to the other strains, showing potent inhibition of *Aspergillus ochraceus* (50.37%). This strain also demonstrated moderate inhibitory effects against *Aspergillus flavus* (23.90%), *Botrytis cinerea* (23.41%), and *Fusarium oxysporum* (16.30%). Additionally, three strains, ~~i.e.~~, *Pseudomonas versuta* R15, *Pseudomonas helleri* R125, and *Pseudomonas trivialis* R150, displayed a highly similar inhibition pattern, effectively suppressing *Fusarium oxysporum* and *Aspergillus ochraceus*, but showing no inhibitory activity against *Aspergillus flavus* and *Botrytis cinerea*. Finally, *Pseudomonas protegens* P79 exhibited vigorous antifungal activity against *Fusarium oxysporum*, with an inhibition rate of 55.56%.

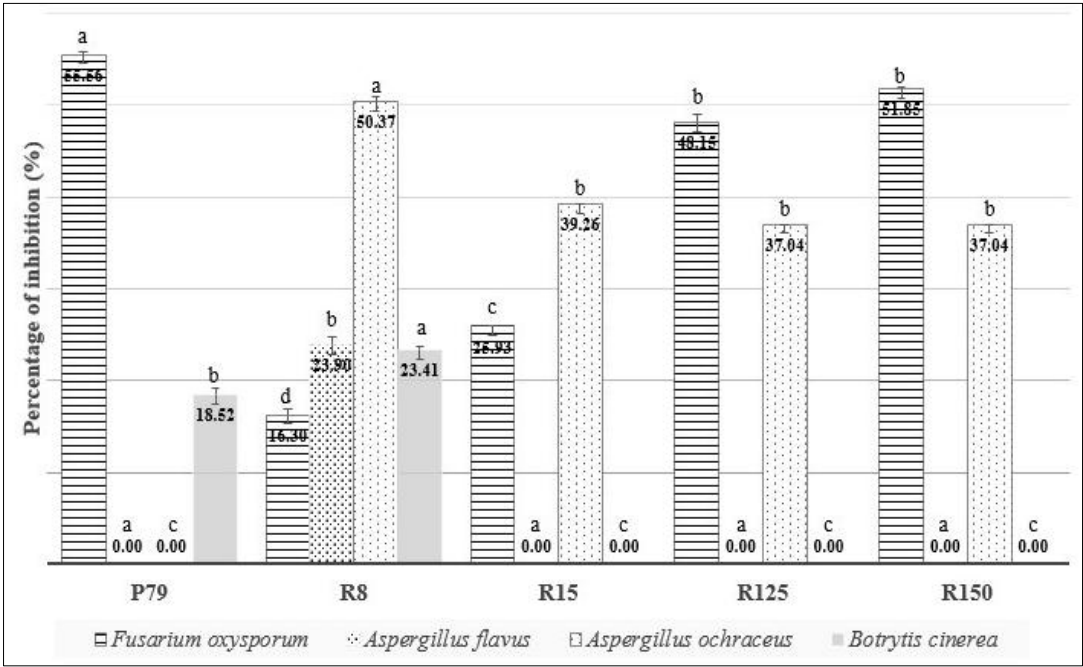


Figure 3: Percentage of growth inhibition of phytopathogenic fungi by *Pseudomonas* strains. Values are expressed as the mean ± SD of three independent replicates (n = 3 pots). Different letters indicate significant differences (ANOVA, $p < 0.05$).

3.7 Stress effect

The resistance,tolerance of the five isolates to salinity, high temperature, and alkaline pH was assessed and is summarized in (Table 5-). Among the five PGPR strains, *Pseudomonas protegens* P79 was the most tolerant, capable of growing at salinity levels up to 13% NaCl, which correlates with its native soil habitat. This strain also tolerated extreme temperatures of up to 45 °C and an alkaline pH of 11. In comparison, *Pseudomonas helleri* R125 and *Pseudomonas versuta* R15 exhibited moderate resistance, growing in the presence of 12% and 11% NaCl, respectively. Both strains also withstood temperatures of 42 °C and

a pH of 10. Among the tested strains, *Pseudomonas trivialis* R150 and *Pseudomonas sesami* R8 showed the lowest salinity tolerance, with growth observed at 9% NaCl, pH 10, and . *Pseudomonas sesami* R8 also exhibited a maximum growth temperature of 37-37 °C. (Table 5).

Table 5. Bacterial tolerance to stress conditions

Strain	Salinity	Temperature	pH
P79	13%	45°C	11
R8	9%	37°C	10
R15	11%	42°C	10
R125	12%	42°C	10
R150	9%	42°C	10

Values are presented as the means of ~~3~~three replicates.

3.8 Inoculation of *Lotus creticus*

The effects of the five rhizobacterial strains on the growth of *L. creticus* plants were investigated ~~(Figures 4 and 5). Results (Figure 4). The results~~ showed ~~significant variation in the~~ that aerial-part length ~~among the~~ was highest in plants inoculated ~~plants, with the highest value recorded in plants treated~~ with *Pseudomonas versuta* R15. ~~A significant reduction compared with the other treatments (Figure 4). In contrast, no significant differences ($P > 0.05$) in root length waswere observed under R150 inoculation compared to~~ between the control and most ~~other treatments.~~ bacterial treatments, except for plants inoculated with R150, which exhibited a lower root length (Figure 4).

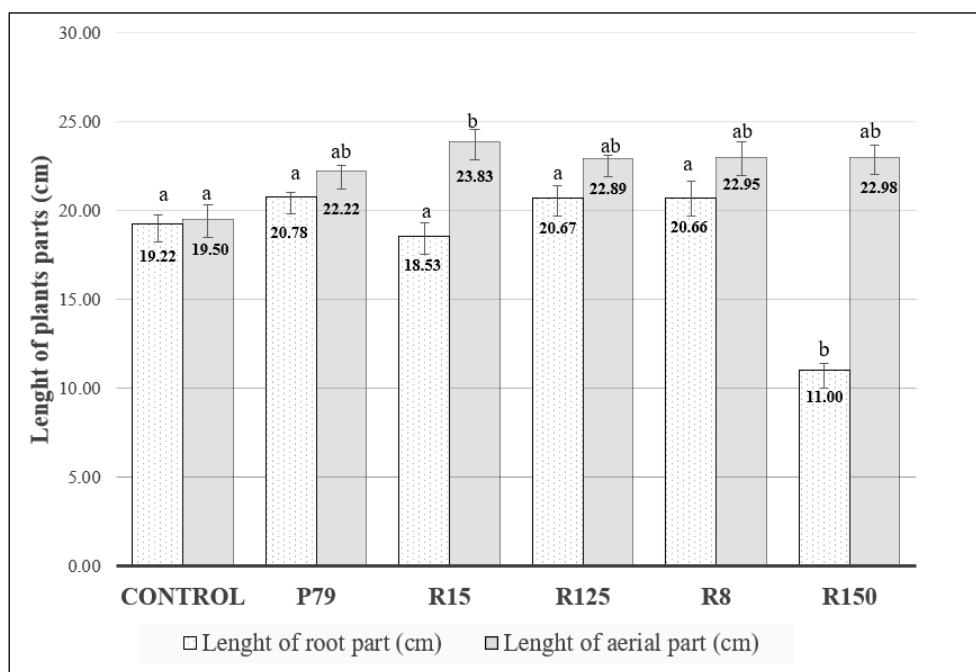


Figure 4: Effect of bacterial strains on the length of root and aerial parts compared to the control. Values are expressed as the mean $\pm$ SD of three independent replicates ($n = 3$ pots). Different letters indicate significant differences (ANOVA, $p < 0.05$).

Additionally, the impact of the five bacterial strains on the fresh and dry weights of both aerial and root parts of *L. creticus* was assessed (is presented in Figure 5). The highest aerial-part dry weight values of aerial parts ($p < 0.05$) were observed in plants inoculated with *Pseudomonas helleri* R125 and *Pseudomonas trivialis* R150, followed by those treated ($P < 0.05$), whereas the highest root dry weight was recorded in plants inoculated with *Pseudomonas protegens* P79 and *Pseudomonas sesami* R8, though to a lesser extent. In contrast, *Pseudomonas protegens* P79 had the most pronounced effect on the root dry weight compared to the control.

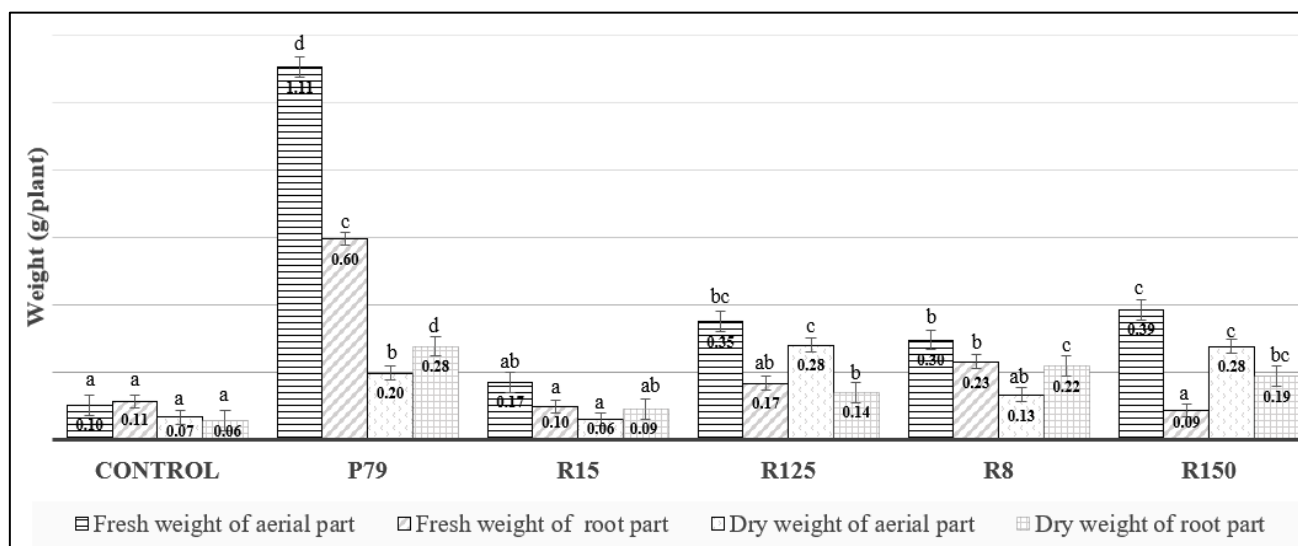


Figure 5: Effect of bacterial strains on the fresh and dry weights of aerial and root parts. Values are expressed as *the mean $\pm$ SD of three independent replicates ($n = 3$ pots).* Different letters indicate significant differences (ANOVA, $pP < 0.05$).

4 Discussion

In Mediterranean coastal regions, *L. creticus* is ~~amongone of~~ the plant species ~~that are~~ well adapted to saline ~~and~~ nutrient-poor environments. Such adaptation is mainly attributed to the ~~capacity of the species~~ *species'* ability to make osmotic and transpiration adjustments, ~~and to~~ produce bioactive compounds, such as hydroxybenzoic ~~acid~~ and gallic acid, ~~which are~~ necessary for maintaining photosynthetic activity under stress conditions (Araniti et al., 2014; Barreira et al., 2017). On the other hand, soil microbiota ~~hashave~~ been extensively reported to play a critical role in enhancing nutrient mobilization and uptake as well as stress tolerance (Howieson et al., 2011; Rima et al., 2018).

In this study, the soil where *L. creticus* was identified was characterized as a sandy Arenosol with an alkaline pH, moderate salinity, and very low levels of organic matter. Such conditions ~~are known to~~ impose multiple constraints on plant growth, including water leaching due to sandy texture, nutrient immobilization and precipitation, under alkaline pH conditions, and osmotic stress associated with salinity (Zhu et al., 2024; Sun et al., 2026). In general, legumes are salt-sensitive, and symbiosis is often limited at high salinity (Bruning and Rozema, 2013). However, ~~the~~ *L. creticus* ~~was able to maintain~~ *maintained* vigorous growth and high ~~capacity of nodulation~~ *capacity*, indicating the functional importance of rhizosphere microorganisms. In this context, five rhizobacterial strains of the genus *Pseudomonas* were isolated from the rhizosphere of *L. creticus* growing wild in the coastal region of Morocco. These strains exhibited notable plant growth-promoting traits that support plant development in environments where nutrient availability is often limited by leaching and ionic imbalances (Sun et al., 2026). The relative contribution of these traits varied significantly among strains, ~~reflecting a synergistic effect~~. At the strain level, distinct

functional profiles indicate that the five *Pseudomonas* isolates may contribute to plant performance through different but complementary mechanisms that collectively support plant establishment and growth in constrained soil environments. Direct effects include plant-level physiological traits, such as IAA production, known to stimulate root system development and improve ~~the plant's capacity to explore the soil~~ soil exploration and nutrient uptake (Etesami and Glick, 2024). ~~Also~~ In addition, ACC deaminase activity plays a key role in alleviating stress-induced ethylene accumulation and mitigating growth inhibition under adverse environmental conditions (Gamalero et al., 2023). In contrast, soil-mediated effects involve processes ~~like~~ such as phosphate solubilization and siderophore-based iron chelation. ~~These effects~~ These effects could also include ammonia production, which affects rhizosphere pH and enhances nutrient solubility and availability (Yang et al., 2025).

Remarkably, all five *Pseudomonas* ~~species~~ strains, particularly *Pseudomonas protegens* P79, ~~were able to~~ could solubilize tricalcium phosphate into soluble forms that can be readily absorbed by plants. Phosphorus is an essential element for plant growth; however, its uptake is often restricted due to its immobilization in soils, particularly sandy soils, where rapid leaching and low organic matter content reduce its uptake by plants (Gerke et al., 2024). In alkaline, calcareous coastal sandy soils, phosphate tends to precipitate by forming insoluble complexes with calcium ions, thereby decreasing its bioavailability (Johan et al., 2021). ~~Increasing~~ Enhanced phosphate solubilization ~~not only enhances~~ may support plant growth ~~but while~~ also increases increasing microbial biomass P, as microorganisms rapidly assimilate the released phosphate. This microbial pool ~~acts~~ may function as a temporary reservoir ~~that is, from which phosphorus could~~ gradually made become available to plants and ~~contributes to~~ potentially influence phosphorus ~~cycling~~ dynamics at the soil–root interface. Additionally, the increase in dry weight observed with *Pseudomonas protegens* P79 ~~may~~ might be ~~attributed~~ related to its ~~metabolic versatility and its ability to produce a wide array of extracellular enzymes, which facilitate~~ broad enzymatic capacity, which facilitates organic matter decomposition and nutrient release under nutrient-limited conditions ~~(Daunoras et al., 2024), but also, as well as~~ to its ability to synthesize phytohormones such as IAA– (Daunoras et al., 2024).

Likewise, the *in vitro* analysis revealed that all selected strains, except *Pseudomonas versuta* R15, produced ~~siderophore compounds~~ siderophores, which are known to enhance iron uptake (Kong et al., 2014). In sandy soils with low organic matter content, iron is commonly present as insoluble Fe³⁺ oxides, which limit its uptake by plants (Singh et al., 2022; Yamagata et al., 2022). Iron deficiency can affect various physiological processes (Singh et al., 2022). In response, many siderophore-producing rhizobacteria can chelate these Fe³⁺ forms and mobilize them into soluble complexes that enhance iron bioavailability (Yamagata et al., 2022). In this regard, the results from the pot trial indicated that *Pseudomonas helleri* R125 and *Pseudomonas trivialis* R150 markedly improved the shoot and root dry biomass of *Lotus* L. creticus, ~~likely due to~~ suggesting a potential contribution of their ~~capacity to mobilize nutrients from the soil~~ nutrient-mobilizing activities, particularly those related to phosphorus and iron. In contrast, ~~inoculation of L. creticus with Pseudomonas versuta R15 led to~~ promoted shoot elongation in L. creticus while resulting in comparatively lower dry biomass ~~but greater shoot length~~.

suggesting a growth-promoting effect that ~~this strain promotes cell elongation, likely through hormonal effects rather than preferentially favored shoot elongation over~~ biomass accumulation (Kurepa et al., 2026).

Also, *Pseudomonas sesami* R8 was able to promote the root dry weight of *L. creticus*, ~~underseering its~~ indicating a potential role in nutrient ~~eyeling (Kumar mobilization (Madhaiyan et al., 2021~~2017). However, the strain exhibited relatively low tolerance to abiotic stress ~~in vitro~~ compared with other *Pseudomonas* species. ~~This suggests, suggesting~~ that ~~its~~ plant growth ~~promotion is promoting effects may be~~ primarily ~~driven by associated with~~ functional traits, such as phytohormone production and nutrient solubilization, ~~which can stimulate root development~~ independently of ~~its~~ stress tolerance (Glick, 2012). Although the strain ~~shows exhibited~~ limited stress tolerance under *in vitro* conditions, it ~~can still exert beneficial effects could potentially~~ contribute to plant performance *in vivo* through plant-associated ~~protection and microbial~~ interactions (Compant et al., 2010; Song et al., 2025). ~~Additionally, secondary~~Secondary metabolites associated with biocontrol could also contribute to improved rhizosphere competence and root colonization ~~efficiency~~ (Lugtenberg and Kamilova, 2009). Notably, *Pseudomonas sesami* R8 demonstrated strong antifungal activity against all tested fungi. ~~A particular This effect that could be attributed is likely~~ attributable to the ~~strain's ability of the strain~~ to produce secondary metabolites, such as ammonia and HCN, which restrict fungal growth, reduce their access to soil nutrients, and enhance rhizosphere health (Sehrawat et al., 2022). These ~~findings observations may~~ suggest a functional bias of *Pseudomonas sesami* R8 toward biocontrol rather than abiotic stress tolerance (Sehrawat et al., 2022). In practice, the strain could be applied as a seed coating or soaking treatment to protect *L. creticus* seeds, suppress fungal colonization, and enhance early plant development. Alternatively, ~~they it~~ could be introduced directly into the rhizosphere through soil inoculation (Paravar et al., 2023). For foliar pathogens such as *Botrytis cinerea*, spraying bacterial suspensions onto leaves may inhibit spore germination and lesion development (Roca-Couso et al., 2021).

~~Overall, these~~

These findings demonstrate the functional diversity of rhizosphere-associated *Pseudomonas* strains and their potential importance in supporting plant establishment and resilience under the combined abiotic stresses of the coastal environment. Most importantly, the ability of strains to grow under high salinity conditions, often associated with arid and semi-arid coastal regions, indicates their capacity to remain metabolically active in saline soils (Wang et al., 2025). However, the plant growth-promoting traits were mainly evaluated in vitro, which may not fully reflect natural soil conditions. Moreover, the underlying mechanisms were inferred from functional assays rather than confirmed by molecular or genomic analyses, and the long-term persistence of the strains was not assessed. Future studies should therefore include field trials across diverse coastal soils, larger sample sizes, and genomic approaches to clarify the mechanisms involved and support the development of effective and sustainable bioinoculant formulations.

5 Conclusion

This study indicates that five *Pseudomonas* strains, particularly *Pseudomonas protegens* P79, originating from Mediterranean coastal soils, can tolerate high salinity and temperature ~~while enhancing and exhibit~~ important ~~soil functions~~functional traits, such as phosphorus solubilization, ~~iron availability, and rhizosphere enzymatic activity. Taken together and siderophore~~

460 **production. Overall**, the findings emphasize the important role of diverse PGPR communities in ~~sustainingsupporting~~ nutrient ~~eyelingavailability~~ and soil fertility in saline, nutrient-poor soils and in supporting plant adaptation to the physicochemical constraints of coastal ecosystems. Nevertheless, further field-based studies, including single or co-inoculation trials, are required to evaluate their effectiveness in enhancing plant growth, nutrient acquisition, and resilience under natural conditions.

Author contributions

465 All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Imane Achkouk and Abdelhay Arakrak. The first draft of the manuscript was written by ~~Imane~~ Imane Achkouk. ~~Anass El Yemlahi contributed to the revision~~ and ~~all authors commented on previous versions~~ the correction of the manuscript. All authors read and approved the final manuscript.

Competing interests

470 The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Data availability

The data used in this study are available from the corresponding author upon reasonable request.

Code availability

No code was used to generate or analyze the data in this study.

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