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Research Article

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Use of *rpoB* gene phylogenetic marker-based distinction of abiotic stress tolerant and plant-growth promoting *Bacillus paralicheniformis* isolates from their closely related *Bacillus licheniformis*

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Abstract

Bacillus paralicheniformis is a new identified species, which was distinguished from *Bacillus licheniformis* in 2015 through extensive phylogenomic and phylogenetic analyses. In this context, this study aimed to achieve a clear identification of the active plant-growth promoting rhizobacteria (PGPR) *B. paralicheniformis* isolates among the closely related *B. licheniformis* through molecular typing, helping for the development of clearly-identified PGPR isolates to be used as biofertilizers. A total of 15 rhizobacteria were isolated from the olive rhizosphere soils. These bacterial isolates exhibited various proprieties in terms of abiotic stress tolerance, biofilm formation under stress conditions, and enzyme activities (*i.e.*, lipases, cellulases, and proteases). In addition, several PGP traits such as phosphate solubilization and the production of siderophores and indol-3 acetic acid were also observed. Molecular identification through 16S rRNA sequencing initially identified all the isolates as *Bacillus* spp. The multi-locus sequence typing (MLST) scheme, using six housekeeping genes (*adk*, *ccpA*, *recF*, *rpoB*, *sucC*, and *spo0A*) had unveiled distinct allelic profiles resulting in 13 unique sequence types (ST). Notably, a comprehensive analysis indicated no exact allele matches existed between the examined isolates and those documented in the PubMLST database. Among the six housekeeping genes, we noticed that *rpoB* gene (RNA polymerase, subunit beta) had multiple polymorphic sites that were bordered by conserved sequences.

Furthermore, the phylogenetic analysis based on *rpoB* had identified clusters indicating distinct phylogenetic relationships between *B. paralicheniformis* and *B. licheniformis*, and successfully differentiated the two species from a pool of 90 strains. The *rpoB* partial sequence proved to be effective for accurate species discrimination.

Keywords: *Bacillus paralicheniformis*, *Bacillus licheniformis*, *rpoB* gene, PGPR, MLST

1. Introduction

The rhizosphere encompasses the soil area directly influenced by the plant roots; typically extending a few millimeters from the root surface. This soil zone harbors significantly higher bacterial populations compared to the adjacent bulk soil. Molecular studies have indicated the presence of over 4,000 microbial species per gram of soil within the rhizosphere (Khade and Sruthi, 2024). Microorganisms residing in the plant rhizosphere possess the capacity to notably augment the plant growth and ameliorate the various challenges encountered in the modern agriculture, thus presenting a promising pathway towards achieving agricultural sustainability (Hakim *et al*., 2021). The primary mechanisms through which these plant growth-promoting rhizobacteria (PGPR) enhance the plant growth include the associative nitrogen fixation, ethylene level reduction, production of siderophores and phytohormones, induction of resistance against pathogens, nutrient solubilization, promotion of mycorrhizal symbiosis, and alleviation of the soil pollutant toxicity (Ashajyothi *et al*., 2024; Khade and Sruthi, 2024). In this respect, the cultivation and production of olives and olive oil hold profound significance within the Moroccan and the broader Middle East and North Africa (MENA) regions. Morocco, in particular, holds a prominent position as one of the world's leading olive oil producers (Ater *et al*., 2016). The genus *Bacillus* plays a pivotal role in the rhizosphere of the olive trees, underscoring their profound importance within this ecosystem. This genus comprises a diverse array of bacterial species known for their versatile traits and multifaceted contributions to the plant health within the olive rhizosphere (Bennis *et al*., 2022).

Bacillus paralicheniformis, a species within the genus *Bacillus*, is a Gram-positive, facultatively anaerobic, motile, rod-shaped, and endospore forming bacterium (Dunlap *et al*., 2015). This species was previously referred to *B. licheniformis* and has recently been separated and described as a new species, based on the phylogenetic and phylogenomic studies (Ashajyothi *et al*., 2024). The genus *Bacillus* is recognized for its large use and effectiveness in producing various biochemicals and molecules that are crucial in the biotechnological industries, such as the enzymes (*i.e.*, proteases, lipases, etc.) (SanthaKalaikumari *et al*., 2021; Zhao *et al*., 2021) and antibiotics (Hakim *et al*., 2021; Khade and Sruthi, 2024). It can also act as an important PGPR (Patani *et al*., 2023) and is able to function as bio-fertilizer and biocontrol agent against the plant pathogenic bacteria (Foysal and Lisa, 2018; Zicca *et al*., 2020) or fungi (Cheffi Azabou *et al*., 2020; Mohamed *et al*., 2019). Phylogenetic comparison of the 16S rRNA gene sequence of the type strain *B. paralicheniformis* KJ-16T with *B. licheniformis* DSM 13T showed 99.4 % sequence similarity (Dunlap *et al*., 2015). Hence, there is an urgent necessity for distinct differentiation of *B. paralicheniformis* from its closely related counterpart, *B. licheniformis*. This differentiation is facilitated by the presence of a potent factor; mainly urease activity, which is a characteristic feature of *B. paralicheniformis* (Ashajyothi *et al*., 2024). Urease activity has been described as a phenotypic criterion enabling the discrimination between these two bacterial species (Dunlap *et al*., 2015) until Jeong *et al*., (2018) disclosed that the urease activity of *B. paralicheniformis* is not a reliable discriminator between these two species. Strains with the same urease gene cluster have been identified in *B.*

licheniformis and *B. sonorensis*, which are the closest relatives of *B. paralicheniformis* (Jeong *et al*., 2018). In addition, specific genetic markers such as the fengycin operon (*fenC* and *fend*) were employed for their differentiation (Olajide *et al*., 2021). Nevertheless, it is crucial to monitor the potential horizontal transfer of fengycin-specific operon genes between *B. paralicheniformis* and *B. licheniformis*, which is often linked with the recombination events, to maintain the validity of these markers (Du *et al*., 2019). As a result, these closely related bacterial species are frequently misidentified due to their significant sequence similarity. The objective of this study was to describe the use of *rpoB* gene phylogenetic based approach as a unique molecular marker employed in the discrimination of the rhizobacterial taxa *B. licheniformis* and *B. paralicheniformis*, which enables an accurate discrimination between these two bacterial species. Our findings also hold the potential to provide fundamental insights into the attributes of *B. paralicheniformis* as a highly abiotic stress tolerant and active PGPR.

2. Materials and methods

2.1. Sampling and bacterial isolates conservation

Soil and root samples were collected from the rhizosphere of olive cultivars located in pedoclimatic regions of southern Morocco (30° 15’ N, 5° 40’ W and 30° 15’ N, 5° 41’ W), which were renowned for olive cultivation. These olive groves were selected from small farms that had never received chemical treatments or synthetic inputs. During the sampling process, the soil and roots were gathered from the base of the olive trees after removing approximately 15 cm of the topsoil layer. These root and soil samples were then carefully placed into sterile bags and transported to the microbiology laboratory for isolation of the viable bacteria. The bacterial isolates were obtained through a serial dilution technique that was conducted by Lahsini *et al*., (2022). Then, detailed characterization of these isolates encompassing the evaluation of their capacity to produce catalase and oxidase enzymes was conducted (Chouati *et al*., 2023). Additionally, their Gram stain reaction and cellular morphology were thoroughly examined using light microscopy. The purified bacterial isolates were subsequently preserved in a solution containing 20 % (v/v) glycerol and Tryptic Soy Broth (TSB) and were stored at -80 °C for future use.

2.2. Characterization of the rhizobacterial isolates

For all the subsequent experiments, the bacterial inocula were prepared from fresh overnight cultures of the purified bacterial isolates by inoculating a single purified colony into 5 ml of TSB followed by incubation in a rotary shaker with agitation at 150 rpm at 28 °C. All experiments were carried out twice in triplicates.

2.2.1. Abiotic stress tolerance

The PGPR isolates exhibit considerable diversity and their efficacy in promoting the plant growth may be highly species-specific among the rhizobacteria. Thus, the currently selected isolates should demonstrate the capability to mitigate the abiotic stress and enhance the plant growth, in order to be used as efficient candidates for biofertilizers conception. The capacity of the rhizobacterial isolates to withstand the drought stress conditions was evaluated by manipulating water activity (A*w*), in reference to Hallsworth *et al*., (1998). This manipulation involved the addition of D-sorbitol (Merck Millipore) to TSB, where the level of A*w* reduction in the TSB was directly proportional to the concentration of the added D-sorbitol. Briefly, a concentration of 520 g/ l D-sorbitol (55 %) was used in TSB resulting in an A*w* value of 0.912. The experiments were conducted in 48-well plates, with each well was inoculated with 10 µl of an overnight bacterial culture, and then the plates were incubated at 28 °C for 48 h with continuous agitation at 180 rpm. To estimate the bacterial growth, the optical density (OD) was quantified spectrophotometrically at OD_{600} nm using a Micro Plates Reader (Epoch 2 BioTek, USA). A non-inoculated TSB medium served as the blank.

The temperature and salinity tolerance were assessed using the viable cell count method conducted by Lahsini *et al*., (2022). To determine the salinity tolerance, varying concentrations of NaCl were incorporated into Tryptic Soy Agar (TSA), which ranged from 0 to 15 % (w/v). For the temperature tolerance assay, 100 µl of the bacterial cultures were inoculated individually into TSA medium and subsequently incubated for 48 h at temperatures that ranged from 10 to 55 °C.

2.2.2. Evaluation of the plant-growth promoting proprieties

The biofilm-forming capacity of *B. paralicheniformis* isolates was evaluated under stress conditions (A*w* 0.912, induced by 55 % D-sorbitol) following the colorimetric protocol described by Esmael *et al*., (2021). For the control group without stress, 100 µl of each fresh culture were inoculated individually into each well of a 48-well microplate containing TSB medium. Under stressful conditions, the TSB medium was supplemented with 550 g/ l of D-sorbitol or 10 % NaCl. The quantification of biofilms formation was performed by measuring the OD at 570 nm after incubating the microplate under static conditions for 24 h at 28 °C. The production of Indole-3-acetic acid (IAA) was assessed following the method described by Chaudhary *et al*., (2021). This procedure involved inoculating 100 µl of the each bacterial isolate into tubes containing 5 ml of TSB that were supplemented with 1 mM L-tryptophan at 28 °C for 3 d with continuous shaking at 150 rpm. Following incubation, 2 ml of each bacterial supernatant that was obtained after centrifugation of the TSB culture for 15 min. at 1000 g were combined with an equal volume of Salkowski reagent; consisting of 12 g/ l of $FeCl_3$ in 7.9 M H_2SO_4. The resulting mixture was then incubated in darkness for 30 min. at room temperature. Subsequently, the OD of the generated pink coloration was measured using a spectrophotometer at 535 nm. This measurement facilitated the calculation of IAA concentration based on a prepared standard curve using known IAA concentrations. To establish a negative control, 2 ml of un-inoculated TSB were mixed with 2 ml of Salkowski reagent. Phosphate solubilization capability was detected on Pikovskaya agar medium that was composed of (g/ l): 0.5 yeast extract, 10 dextrose, 5 calcium phosphate, 0.5 ammonium sulfate, 0.2 potassium chloride, 0.1 magnesium sulfate, 0.0001 manganese sulfate, 0.0001 ferrous sulfate, 15 agar, dissolved in 1 l of dist. water (Pande *et al*., 2017). After solidification, 100 µl of each fresh bacterial suspension was spot inoculated individually onto the surface of Pikovskaya medium followed by incubation at 28 °C for 7 d. The formation of a solubilization halo around the bacterial colony indicated a positive result. The production of siderophores, encompassing both hydroxamate and catecholate types, was quantified following the procedure outlined by Carson *et al*., (1992). Approximately 1 ml of the bacterial supernatant (prepared as indicated before) was mixed with 3 ml of 2 % aqueous $FeCl_3$ solution, followed by subjecting the resulting mixture to a spectrophotometric analysis. The presence of hydroxamate-type siderophores was determined by monitoring the development of a brown color at 430 nm, while the assessment of catecholate was carried out by detecting the formation of wine-colored complex at 495 nm. The blank treatment consisted of 1 ml of dis. water and 3 ml of 2 % aqueous $FeCl_3$ solution. The production of hydrolytic enzymes was evaluated following the methods described by Chouati *et al*., (2023) for lipases and proteases, and Ngom *et al*., (2023) for cellulases. For lipolytic activity assessment, nutrient agar (NA) medium supplemented with 10 ml of Tween 80 as a lipid substrate was utilized. Following the bacterial culture inoculation in the form of a longitudinal streak, the medium was incubated at 28 °C for 48 h. The presence of a precipitation zone around the streak indicated the production of lipases by the rhizobacterium (Chouati *et al*., 2023). The milk agar medium that was supplemented with 100 g of skim milk was used to evaluate the proteolytic activity. The bacterial isolates were streak inoculated individually onto the milk agar plates and incubated at 28 °C for 48 h. A positive reaction was identified by the formation of a distinct

zone of proteolysis around the bacterial streak (Chouati *et al*., 2023). To quantify the cellulase activity, a basal salt medium containing carboxymethyl cellulose (CMC) as the sole carbon source was used following the chemically defined components described by Ngom *et al*., (2023). 100 µl of each bacterial culture were inoculated individually on the agar medium and then incubated at 28 °C for 48 h. After incubation, the formation of hydrolysis zones was visualized using the Congo red method after consecutive treatments with a 0.1 % Congo red solution for 20 min. each, followed by a rinse with 1 M NaCl for 20 min.

2.3. Molecular characterization of the rhizobacterial isolates

2.3.1. DNA extraction and polymerase chain reaction (PCR)

The genomic DNA extraction was carried out using the BIOLINE extraction kit (Meridian Bioscience, UK), according to the manufacturer's instruction. The full 16S rRNA coding gene (~1500 base pairs) was amplified by employing the universal primer pair: *fD1* (5'-AGAATTTGATCTTGGTTCAG-3') and *rP2* (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg *et al*., 1991). While, the Multi-Locus Sequencing Analysis (MLST) primers used in this study are listed in Table (1). The PCR reaction was carried out in a 25 ml reaction mixture for each sample using the KAPA kit (KAPA Biosystems). The mixture contained 0.50 µM of each primer, 0.20 mM of dNTP mix., 5 µl of KAPA2G Buffer (1X), 0.50 U of Taq polymerase, and 1 µl of template DNA. The final volume was adjusted with dist. water. The PCR program included initial denaturation at 95 °C for 2 min., 25 cycles of denaturation at 95 °C for 30 sec., annealing for 30 sec., and extension at 72 °C for 30 sec. with a final extension at 72 °C for 30 sec. The amplified PCR products were electrophoresed on a 1 % agarose gel, stained with Ethidium bromide referring to a GeneRulerTM 1kb DNA ladder, and then visualized using the Gel Doc imaging system and Image Lab software (Bio-Rad, USA).

2.3.2. Sequencing

Purification of PCR products was performed using the ExoSAP-IT purification kit (Thermo Fisher Scientific, Waltham, MA). Sequencing was executed using the Big Dye Terminator Cycle Sequencing v3.1 kit (Applied BioSystems, CA, USA) in both forward and reverse directions with the same primers used for PCR, following the manufacturer's guidelines. Then, the sequences data were collected on an ABI Prism 3130XL genetic analyzer (Applied BioSystems, CA, USA).

2.3.3. Phylogenetic analysis and molecular typing

The obtained 16S rRNA sequences were processed and assembled using DNA Baser Sequence Assembler software (V5.15). Subsequently, to facilitate identification and comparative analysis against the reference datasets, the BLAST algorithm integrated within the EzBioCloud 16S rRNA database was employed (Yoon *et al*., 2017). Additionally, the MLST gene sequences were analyzed following the PubMLST (https://pubmlst.org/) scheme, generating sequence type (ST) data for each bacterial isolate. Phylogenetic analyses were conducted by constructing four trees by MEGA XI software using distinct sequence datasets. Firstly, a phylogenetic tree was constructed using the sequences of the 16S rRNA gene. The second and third trees were constructed using concatenated sequences of the six housekeeping genes (HKG) and an individual *rpoB* gene only, respectively. Lastly, a separate tree was generated focusing on *rpoB* gene using 90 isolates, where 15 isolates were from this study and 75 belong to *B. licheniformis* (50) and *B. paralicheniformis* (25) retrieved from the PubMLST database.

2.4. Statistical analysis

Statistical significance was assessed using ANOVA, followed by a Tukey's HSD test, to determine the significance of differences between the replicates across the studied parameters (*p*-value <0.05).

Table 1: List of PCR primers used for amplification of the six house-keeping genes

Gene	Target gene	Forward primer 5'-3'	Reverse primer 5'-3'	Reference
adk	Adenylate kinase	GGTAAAGGGACACAGGCTGA	TCGAGTAAAGGCTGGGTTTG	(Madslien *et al*., 2012)
ccpA	Transcriptional regulator	TATGATGTAGCACGCGAAGC	TATCCCCAAGCGCTCTTTTA	(Madslien *et al*., 2012)
recF	Recombination protein F	ACGGTTCTGTTCCCATTCAG	CATCACGGCCATTGACATAG	(Madslien *et al*., 2012)
rpoB	DNA-directed RNA polymerase, subunit beta	GGGTCCCGACGG CCAACAAA	GGCCGGTTCCCCTCCGTAGT	(De Clerck and De Vos, 2004)
spo0A	Succinyl-CoA synthetase, subunit beta	AGGTCAACTAGTTCAGTATGGACG	AAGAACCGTAACCGGCAACTT	(Madslien *et al*., 2012)
sucC	Transcriptional regulator	GAAGTGCTTGGTGTCGCATA	TGTGTAGCCGAAAAGTGACG	(Madslien *et al*., 2012)

3. Results

A total of 15 rhizobacterial isolates were obtained from the olive rhizosphere soils. Each of these bacterial isolates was assigned a label beginning with "OZ" followed by a numerical identifier. All isolates displayed a Gram-positive reaction and had rod-shaped cell arrangements. Furthermore, they exhibited positive results for both of the catalase and cytochrome oxidase enzymes, which were observed through the formation of gas bubble around the bacterial colonies and a transition in the color of the oxidase discs to violet, respectively.

3.1. Tolerance to the abiotic stresses

The tolerance to abiotic stress capabilities of *B. paralicheniformis* isolates' are detailed in Table (2). Regarding the salinity tolerance, most isolates exhibited the ability to grow at NaCl concentrations up to 10 %. Notably, isolate OZ-5 demonstrated robust growth even in the presence of 15 % NaCl. Concerning the temperature tolerance, all bacterial isolates exhibited psychrotolerant and thermotolerant capabilities, demonstrating their ability to thrive across a broad temperature range spanning from 15 to 55 °C. Meanwhile, for the drought stress tolerance, all 15 tested isolates showed consistent tolerance to a concentration of 520 g/ l of D-sorbitol, which is equivalent to an A*w* of 0.912. Notably, eight of the rhizobacterial isolates, including OZ-48, OZ-69, OZ-76, OZ-77, OZ-80, OZ-87, OZ-92, and OZ-94, displayed highly tolerance characteristics.

3.2. Biofilm formation capacity

The rhizobacterial isolates demonstrated an adaptive response to the diverse stress conditions by creating biofilms, which is a significant survival strategy in the plant rhizosphere. Fig. (1) illustrates this adaptation, where the absorbance values at OD_{570}nm were employed to assess the biofilm formation activity of the 15 *B. paralicheniformis* isolates, under both of NaCl and D-sorbitol induced stresses. This suggests that the biofilm-forming capacity remains effective under several stressful conditions either of salinity (10 % NaCl) or drought. The results indicated a decline in the bacterial biofilm formation activity when exposed to drought stress. Three bacterial isolates; mainly OZ-77, OZ-92, and OZ-48 displayed the highest level of biofilm formation in response to drought stress conditions, with an optical density (OD_{570}nm) of 1.8, 1.7 and 1.6; respectively, while the OZ-90 and OZ-77 isolates expressed the highest salt stress tolerance (OD_{570}nm of 0.95 and 0.82, respectively).

Table 2. Morphological, biochemical, and abiotic stress tolerance assays of the 15 *B. paralicheniformis* isolates

Isolate	Shape/ Gram reaction	Biochemical assays		NaCl		Aw 0.912	Temperature tolerance	
		Catalase	Oxidase	10 %	15 %		15 °C	55 °C
OZ-5	Rod shape/+	+	+	HT	T	0.41± 0.09	T	HT
OZ-14	Rod shape/+	+	+	HT	S	0.44± 0.05	T	HT
OZ-15	Rod shape/+	+	+	HT	S	0.40±0.07	T	HT
OZ-44	Rod shape/+	+	+	HT	S	0.48±0.08	T	HT
OZ-48	Rod shape/+	+	+	HT	S	0.63±0.03	T	HT
OZ-69	Rod shape/+	+	+	HT	S	0.52±0.10	T	HT
OZ-71	Rod shape/+	+	+	HT	S	0.40±0.03	T	HT
OZ-72	Rod shape/+	+	+	HT	S	0.41±0.04	T	HT
OZ-76	Rod shape/+	+	+	HT	S	0.50±0.03	T	HT
OZ-77	Rod shape/+	+	+	HT	S	0.67±0.02	T	HT
OZ-80	Rod shape/+	+	+	HT	S	0.53±0.03	T	HT
OZ-87	Rod shape/+	+	+	HT	S	0.54±0.02	T	HT
OZ-90	Rod shape/+	+	+	HT	S	0.49±0.09	T	HT
OZ-92	Rod shape/+	+	+	HT	S	0.52±0.02	T	HT
OZ-94	Rod shape/+	+	+	HT	S	0.51±0.03	T	HT

Where: Numerical data are represented as means of three replicates (±) Standard deviation. S: Sensitive; T: Tolerant; HT: highly tolerant; +: positive; -: negative result

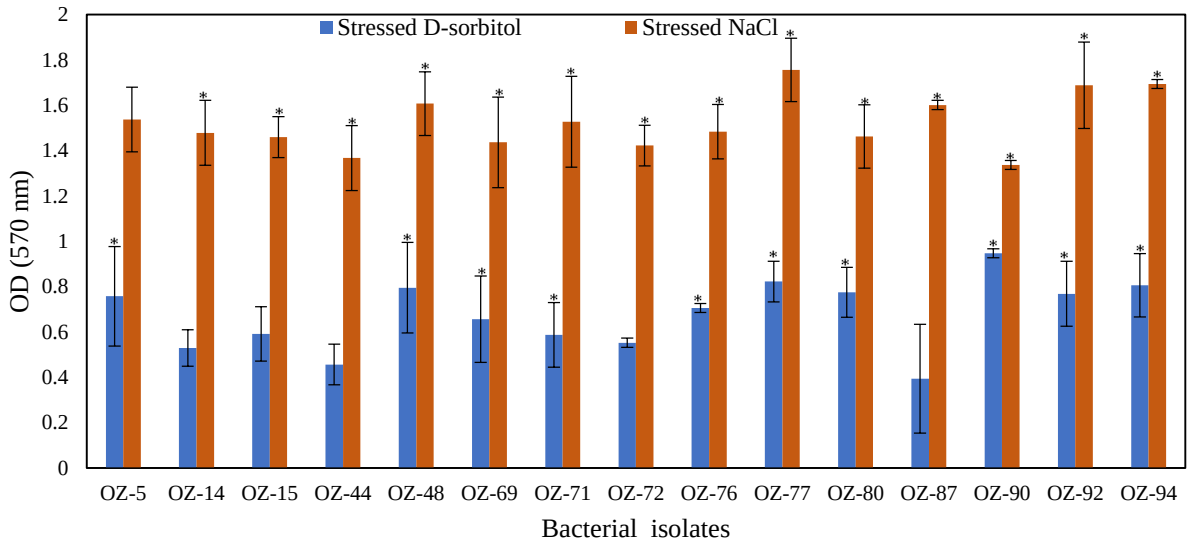


Fig. 1. Quantitative assessment of biofilm formation by *B. paralicheniformis* isolates, which was conducted under stressful conditions of D-sorbitol (55 %) and NaCl (10 %). The error bars followed by (*) show a statistically significant difference ($p < 0.05$) with the control plates (not exposed to the bacterial inocula)

3.3. Plant growth promoting attributes of the bacterial strains

The 15 *B. paralicheniformis* isolates were subjected to a comprehensive analysis of various plant growth promoting (PGP) traits, including IAA production, phosphate solubilization, siderophore formation, and extracellular enzyme activity (Table 3). The results highlighted the notable capacity of these bacterial isolates to produce indole-type molecules from L-tryptophan, varying between 16 and 25 µg/ ml, with the isolate OZ-48 exhibiting the highest IAA production capability (25.50 µg/ ml) followed by isolate OZ-77 (22.90 µg/ ml). They demonstrated the capacity to solubilize phosphate from an insoluble source, as evidenced by the formation of clear halo zones around the colony's growth (Fig. 2A). The isolates also showed the ability to produce both siderophores-types; hydroxamate and catecholate; with isolate OZ-48 standing out as the most proficient siderophore producer. Quantitative assessment of the extracellular enzyme production revealed the significant lipase activity, which was tested by using Tween 20 as a substrate, where all isolates except for OZ-44 had shown the formation of a precipitation zone around the bacterial streak (Fig. 2B). Meanwhile, significant protease activity in all isolates was observed, which was marked by the formation of a discernible zone surrounding the bacterial streak (Fig. 2C). The tested bacterial isolates displayed significant potential in terms of cellulase enzyme production, showing a clear zone of degradation in the medium containing the CMC substrate (Fig. 2D).

3.4. 16S rRNA based identification of the rhizobacterial isolates

The 15 rhizobacterial isolates underwent molecular characterization through the sequencing of the 16S rRNA gene, and the resulting sequences (approximately 1.5 kb) were assembled and deposited in GenBank NCBI with accession numbers given in Table (4), along with BlastN results obtained by analyzing the sequences in the EzbioCloud database. These rhizobacterial isolates belonged to the genus *Bacillus*, with identity rates ranging from 99 % to 100 %, indicating their affiliations with both of *B. licheniformis* and *B. paralicheniformis*. The genetic relationships were validated through a phylogenetic tree analysis (Fig. 3), revealing one primary clade corresponding to the two mentioned bacterial species.

3.5. Molecular typing

3.5.1. MLST-based discrimination of *B. licheniformis* and *B. paralicheniformis*

Regarding a robust identification of the 15 rhizobacterial isolates that were attributed to the *B. licheniformis*/ *B. paralicheniformis* clade, a more comprehensive characterization was undertaken using MLST typing, involving the sequencing of six HKG genes; mainly *adk*, *ccpA*, *recF*, *rpoB*, *sucC*, and *spo0A*. These genes were meticulously sequenced and subjected to analysis via the PubMLST database. As delineated in Table (5), each isolate was assigned a distinct sequence type (ST) based on the allele's similarity. Noteworthy, certain isolates emerged as novel STs, which were marked by their varying allele disparities. Meanwhile, no instances of complete allele matches were discerned between the analyzed isolates and the references within the database. In effect, ST35 (an isolate with sequence type allele no. 35 in PubMLST database) was the prevailing occurrence, constituting a total of 9 instances, out of a spectrum of thirteen related STs encompassing ST11, ST15, ST17, ST27, ST28, ST33, ST35, ST36, ST37, ST47, ST48, ST49, and ST51. Furthermore, all the documented isolates were classified as *B. paralicheniformis*. This determination is further supported by the results of the phylogenetic tree analysis, where concatenated sequence alignments were used (Fig. 4). The resultant phylogeny was bifurcated into two clusters: the principal cluster features the *B. paral*icheniformis reference strains alongside the 15 isolated rhizobacterial strains, while the secondary cluster accommodated all the other *B. licheniformis* reference strains.

Table 3. The *in vitro* assays of hydrolytic enzyme's activities, phosphate solubilization, and siderophores and indole-3 acetic acid (IAA) production of the tested 15 rhizobacterial isolates

Isolate no.	Extracellular enzymes production			Plant-growth promoting activity			
	Lipase	Cellulase	Protease	Phosphate solubilization	Siderophores		Indole-3 acetic acid (mg/ l)
					Catecholate (OD_{495}nm)	Hydroxamate (OD_{495}nm)	
OZ-5	+	+	+	+	1.098±0.19	3.061±0.22	17.50±0.14
OZ-14	+	+	+	+	0.844±0.20	2.748±0.08	21.55±0.14
OZ-15	+	+	+	+	0.826±0.14	2.704±0.12	18.90±0.09
OZ-44	-	+	+	+	0.916±0.19	2.853±0.09	22.05±0.14
OZ-48	+	+	+	+	1.293±0.02	3.313±0.20	25.50±0.14
OZ-69	+	+	+	+	0.802±0.14	2.623±0.19	20.25±0.20
OZ-71	+	+	+	+	0.804±0.14	2.720±0.14	22.25±0.20
OZ-72	+	+	+	+	0.827±0.14	2.648±0.02	21.35±0.09
OZ-76	+	+	+	+	0.691±0.08	2.430±0.02	16.95±0.12
OZ-77	+	+	+	+	0.747±0.02	2.518±0.09	22.90±0.14
OZ-80	+	+	+	+	0.895±0.12	2.827±0.11	20.10±0.14
OZ-87	+	+	+	+	0.835±0.17	2.671±0.24	19.6±0.02
OZ-90	+	-	+	+	1.000±0.09	2.897±0.02	16.65±0.02
OZ-92	+	+	+	+	0.641±0.05	2.388±0.14	18.90±0.19
OZ-94	+	+	+	+	0.629±0.12	2.347±0.14	18.50±0.02

Where; The numerical data are represented as means of three replicates (±) Standard deviation (SD). +: positive result; –: negative result; OD: optical density

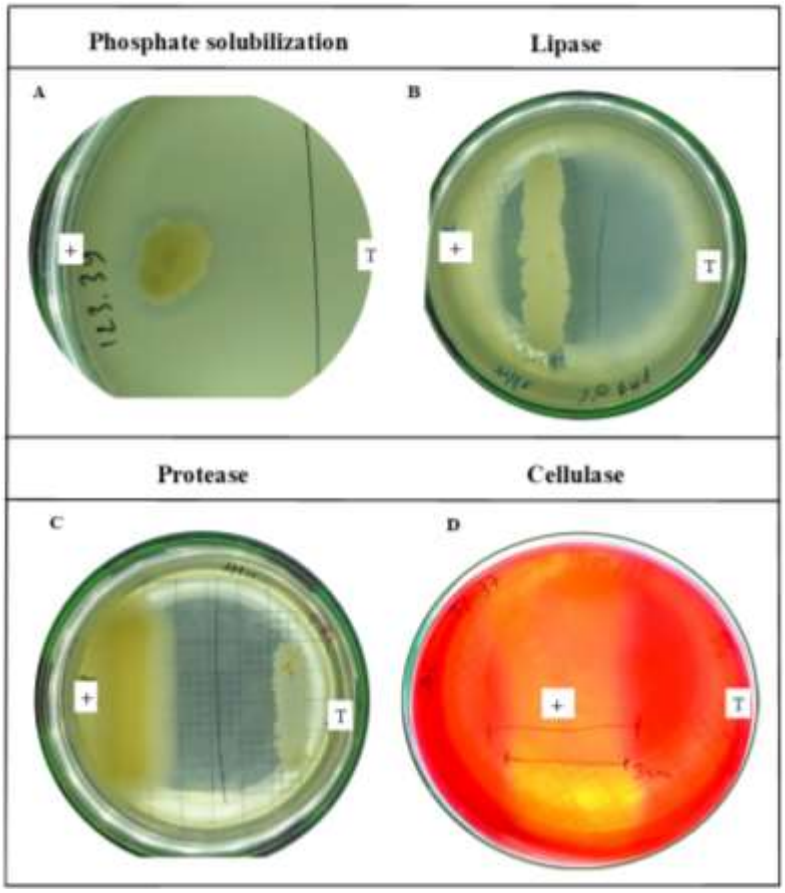


Fig. 2. *In vitro* assays used to evaluate the attributes of *B. paralicheniformis* isolates, for phosphate solubilization (A), and lipase (B), protease (C), and cellulase (D) activities. Where; (+): positive result and (T): negative control

Table 4. Locations, geographical coordinates, and 16S rRNA sequence analysis of the tested 15 rhizobacterial isolates

Isolate	Location			GenBank Accessions No.	16S rRNA identification	
	Altitude (m)	Latitude (N)	Longitude (W)		Closest reference strains	Identity (%)
OZ-14				OP901389	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-44	687	30º 15,311’	5º 40,956’	OP901392	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-48				OP901394	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	100
OZ-5	692	30º 15,099’	5º 40,984’	OP901387	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	100
OZ-76				OP901402	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	100
OZ-77				OP901403	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-80				OP901404	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-87	690	30º 15,441’	5º 41,497’	OP901407	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-90				OP901408	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-92				OP901409	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-94				OP901410	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-15				OP901390	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-69	699	30º 15,385’	5º 41,490’	OP901399	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-71				OP901400	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99
OZ-72				OP901401	*B. licheniformis ATCC 14580^T / B. paralicheniformis KJ-16^T*	99

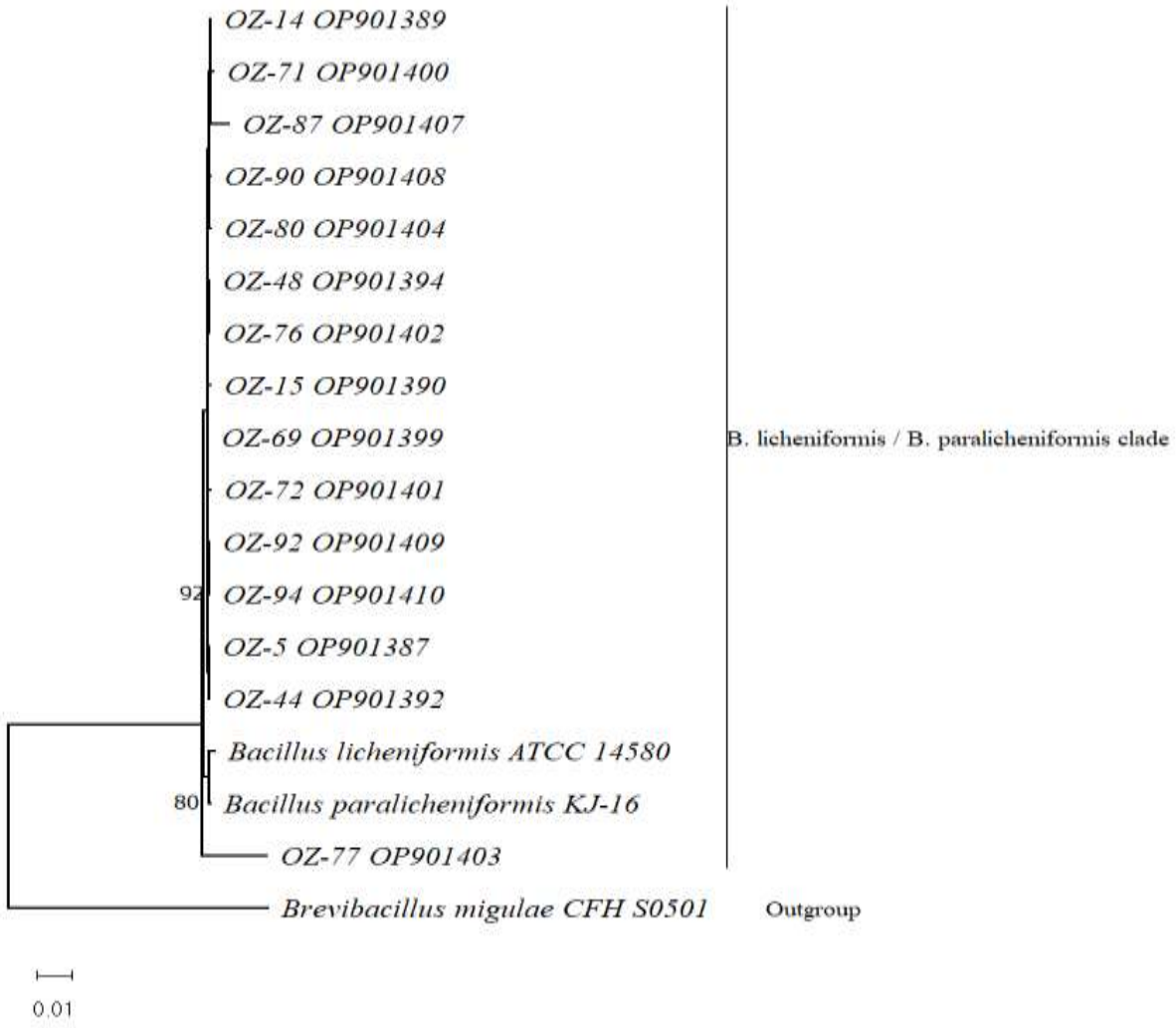
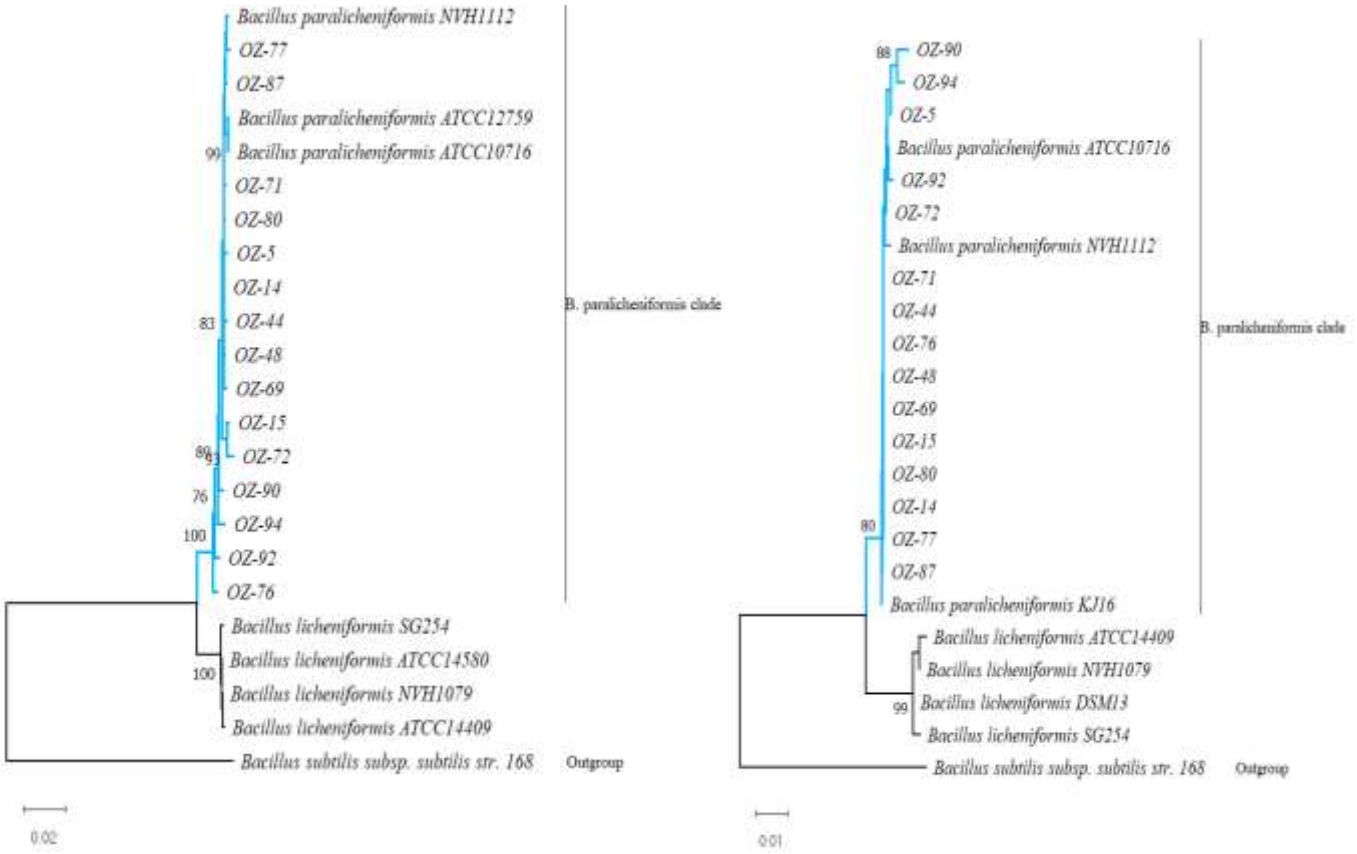


Fig. 3. Phylogram of the 15 rhizospheric isolates, which was constructed using 16S rRNA gene sequences. The Neighbor-joining method was applied, with a bootstrap value of 1000 repetitions. GenBank accession numbers are given ahead of the strain's names, and *Brevibacillus migulae* was used as an outgroup

Table 5. Multi-locus sequence typing (MLST) of the 15 *B. paralicheniformis* rhizospheric isolates, showing differences among the genes, matching alleles, and types of sequences of the closest isolates in PubMLST database

Isolate no.	MLST						Allele	Strains	PubMLST database						
	a	b	c	d	e	f			a	b	c	d	e	f	ST
OZ-5	3	2	5	3	13*	3	5	749	3	2	5	3	8	3	15
							5	NVH800	3	2	5	3	9	3	27
							5	DSM11258	3	7	5	3	13	3	35
							5	CNTS31	3	2	5	3	12	3	51
OZ-14	3	2	5	3	13	3	5	749	3	2	5	3	8	3	15
							5	NVH800	3	2	5	3	9	3	27
							5	DSM11258	3	7	5	3	13	3	35
							5	CNTS31	3	2	5	3	12	3	51
OZ-15	4*	7	5*	3	8*	3	5	NA	3	7	5	3	8	3	49
OZ-44	3*	4	5	3	13	3	5	DSM11258	3	7	5	3	13	3	35
OZ-48	3	2*	5*	3	13	3	5	749	3	2	5	3	8	3	15
							5	NVH800	3	2	5	3	9	3	27
							5	DSM11258	3	7	5	3	13	3	35
							5	CNTS31	3	2	5	3	12	3	51
OZ-69	3	7*	5*	3	13	3	6	DSM11258	3	7	5	3	13	3	35
OZ-71	3	2	5	3	13*	3	5	749	3	2	5	3	8	3	15
							5	NVH800	3	2	5	3	9	3	27
							5	DSM11258	3	7	5	3	13	3	35
							5	CNTS31	3	2	5	3	12	3	51
OZ-72	4*	14*	5*	3	8*	3	4	749	3	2	5	3	8	3	15
							4	DSM11259	3	14	5	3	2	3	36
							4	NA	3	7	5	3	8	3	49
OZ-76	4*	1*	5	3	13	3	4	DSM11258	3	7	5	3	13	3	35
OZ-77	3	7*	5	3	15*	7*	4	CCUG26008	3	7	5	3	7	3	11
							4	NVH1112	3	7	5	5	2	7	17
							4	CHCC15381	3	7	5	3	12	3	33
							4	DSM11258	3	7	5	3	13	3	35
							4	NCTC8233	3	7	5	3	2	3	47
							4	ATCC13438	3	7	5	3	8	3	49
OZ-80	3	2	8	3	9	3	5	NVH800	3	2	5	3	9	3	27
							5	B-14251	3	2	8	3	2	3	37
							5	MB647	3	2	10	3	9	3	48
OZ-87	3	7	5	3	16	3	5	CCUG26008	3	7	5	3	7	3	11
							5	CHCC15381	3	7	5	3	12	3	33
							5	DSM11258	3	7	5	3	13	3	35
							5	NCTC8233	3	7	5	3	2	3	47
							5	ATCC13438	3	7	5	3	8	3	49
OZ-90	4*	2*	8	3*	9	3	4	NVH800	3	2	5	3	9	3	27
							4	B-14251	3	2	8	3	2	3	37
							4	MB647	3	2	10	3	9	3	48
OZ-92	3	9*	8	3	9	3	4	NVH800	3	2	5	3	9	3	27
							4	LMG17662	3	12	6	3	9	3	28
							4	B-14251	3	2	8	3	2	3	37
							4	MB647	3	2	10	3	9	3	48
OZ-94	4*	2*	8*	3*	13*	3	4	B-14251	3	2	8	3	2	3	37

Where; MLST: Multi-locus sequence typing; a: *adk*, b: *ccpA*, c: *recF*, d: *rpoB*, e: *spo0A*, f: *sucC*, ST: sequence type. *: Indicates one or more difference between the allele sequence and the PubMLST reference



(A) **(B)**

Fig. 4. Phylogenetic tree of 15 *B. paralicheniformis* rhizobacterial isolates, created based on the concatenated sequences of six house-keeping genes **(A)** and the *rpoB* partial gene sequence **(B)**. The Neighbor-joining method was employed with 1000 bootstrap repetitions. *Bacillus subtilis* was used as an outgroup

3.5.2. *rpoB* gene based identification

Although MLST is effective in distinguishing between *B. licheniformis* and *B. paralicheniformis*; however, it is not as straightforward and rapid as 16S rRNA sequencing and the other PCR methods. Among the six HKG genes, the partial sequence of *rpoB* gene was identified as the most efficient and conserved gene sequence for discriminating between the two species. In this context, a satisfactory number of polymorphic sites were identified in the *rpoB* gene sequence, thus providing the necessary distinction between the two species. Hence, a phylogenetic tree was constructed using partial sequences of the *rpoB* gene, which demonstrated its efficacy in discerning between *B. licheniformis* and *B. paralicheniformis* (Fig. 4B). Indeed, in the constructed phylogram; two clearly defined clusters were observed, which effectively segregated the *B. paralicheniformis* group from the second clade that encompassed the reference strains of *B. licheniformis*. Furthermore, a Neighbor-Joining phylogenetic tree was generated based on the *rpoB* partial gene sequences for the 90 bacterial strains. The resulting phylogenetic tree clearly delineated the

bacterial strains into two distinct clusters corresponding to the two main rhizobacterial species, *B. licheniformis* and *B. paralicheniformis* (Fig. 5). A separate branch in the phylogenetic tree housed all the reference strains of *B. paralicheniformis* along with the 15 studied rhizobacterial isolates, indicating their strong affiliation within this species. This implies that the *rpoB* gene partial sequences phylogenetic analysis had provided an enhanced discriminatory capability for distinguishing between the two closely related rhizobacterial species.

4. Discussion

The advancement of the molecular taxonomic methods; particularly the phylogenomic approaches, has significantly enhanced the precision of bacterial species discrimination, thus enabling the identification of novel species. This is critical, not only for establishing new taxonomic positions, but also for comprehending the functional characteristics and potential applications of the closely related bacterial species. For instance, the composition of the rhizobacterial communities is influenced by the choice of the fertilizer types in the different agricultural systems (Raimi *et al*., 2023). Moreover, precise identification of *B. paralicheniformis* is particularly relevant due to its potential misidentification with its close relative, *B. licheniformis*. The prevalence of both bacterial species in numerous recent studies underscores their widespread use across various bio-industries, including but not limited to the animal feed, human food; as components in the additives, enzymes, and plant protection products (Maski *et al*., 2021; SanthaKalaikumari *et al*., 2021; Koutsoumanis *et al*., 2023). Upon our analyses, the two isolates; namely *B. paralicheniformis* OZ-48 and *B. paralicheniformis* OZ-77, had stood out prominently in terms of the abiotic stress tolerance, PGP traits, biofilm formation under stress conditions, and enzyme activities. They exhibited impressive halotolerance, through thriving in environments with extremely high salt concentrations, surpassing 10 % (w/v) NaCl. They also displayed a high tolerance feature under drought stress conditions of A*w* 0.912. Such properties have the potential to enhance the plant resistance and productivity in the dry and saline environments (Abbas *et al*., 2019). Furthermore, the two rhizobacterial isolates demonstrated the ability to solubilize phosphate and synthesize siderophores and the phytohormone indole-3-acetic acid; recording 25.50 and 22.90 µg/ml for OZ-48 and OZ-77, respectively. In addition, both rhizobacterial isolates demonstrated significant ability to produce various hydrolytic enzymes, including lipases, proteases, and cellulases. These enzymes are essential components of their biocontrol arsenal in the rhizosphere, which help them to effectively combat the phytopathogens by breaking down the cell wall components of these pathogens (Tian *et al*., 2022). Therefore, these *B. paralicheniformis* rhizobacterial isolates represent a promising approach for the development of bio-fertilizers, aiming to reduce the use of the chemical fertilizers, thereby contributing to the mitigation of the soil degradation and water pollution. In this study, the 15 newly obtained rhizobacterial isolates were initially identified as *Bacillus* spp. (*B. licheniformis* or *B. paralicheniformis*) through 16S sequencing. This suggested that these approaches may not sufficiently differentiate between these bacterial species, a situation that has been observed with *Bacillus* spp. (Figueroa-Brambila *et al*., 2023). Although 16S rRNA gene sequencing has traditionally been considered as the gold standard for bacterial identification and phylogenetic inferences; however, the misidentification of these bacterial species may be attributed to the insufficient sequence variation between the closely related species, as they share more than 99.4 % of sequence similarity (Ashajyothi *et al*., 2024).

The limitations of differentiation via 16S rRNA sequencing prompted the implementation of the MLST approach, which is renowned for its ability to discern the nuanced genetic relationships among the closely related species (Ngom *et al*., 2023).

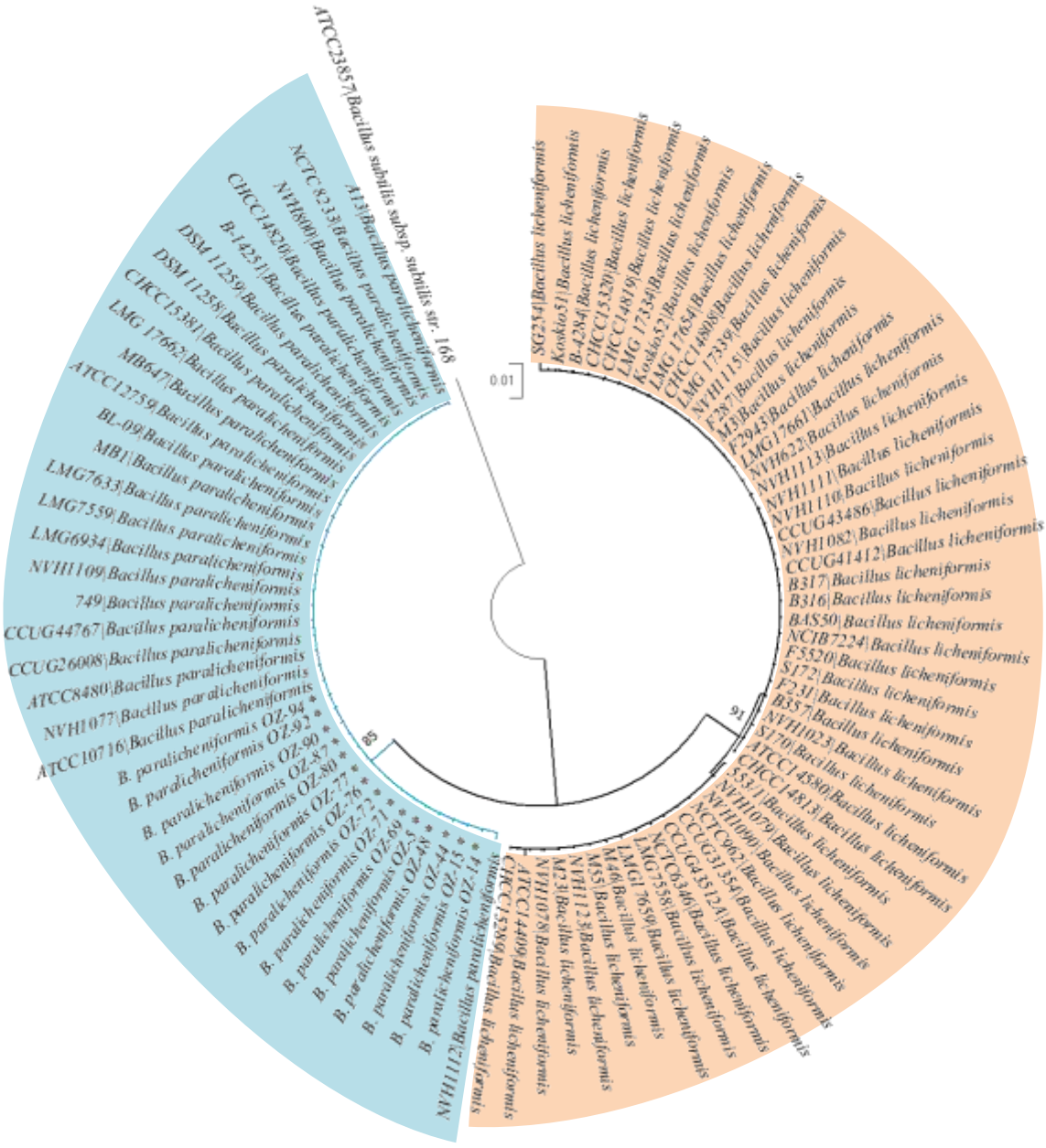


Fig. 5. A phylogenetic tree that has been constructed using the Neighbor-Joining method with 1000 replicates, on the basis of the partial *rpoB* gene sequences of the 15 studied *B. paralicheniformis* rhizobacterial isolates (marked with *); alongside with 50 *B. licheniformis* (purple clade) and 25 *B. paralicheniformis* (blue clade) retrieved from the PubMLST database. *B. subtilis* was used as an outgroup

In the present study, using the MLST technique, the 15 rhizobacterial *Bacillus* isolates were classified as *B. paralicheniformis* species. Interestingly, there was no complete allele match between our isolates and those registered in the PubMLST database that showed different allelic profiles with 13 different STs, which may be attributed to the variations between their activities. These revelations underscore that the diversities in PGPR traits do not seem to adhere to the species-specific patterns. Indeed, several other research works have highlighted the significance of the bacterial strains such as MDJK30 and ES-1 within the *B. paralicheniformis* species, given their utilization in biotechnology and their role as PGPR with the capacity to suppress the phytopathogenic bacteria and fungi (Wang *et al*., 2017; Du *et al*., 2019; Iqbal *et al*., 2022).

Although MLST effectively distinguishes between *B. licheniformis* and *B. paralicheniformis*, but it lacks the simplicity and speed that are inherent in 16S rRNA sequencing and the other PCR methods. This study described the use of the *rpoB* gene partial sequences, limited to 500 bp, as a sufficient proof that enables the differentiation of *B. paralicheniformis* from its related *B. licheniformis*. Hence, the gene sequencing has proven to be the most efficient and conserved method for such discrimination with a significant number of polymorphic sites. The *rpoB* gene encodes for the β-subunit of the RNA polymerase enzyme that is crucial for DNA transcription; an essential cell survival key, which is present in all bacteria in a single copy and is not subjected to horizontal transfer events (Lin *et al*., 2023). The *rpoB* gene has been cited in the literatures as one of the most discriminative genes among the closely related bacterial species (Ogier *et al*., 2019). The *rpoB* gene sequence analysis has been reported to be used for the identification of Elizabethkingia species (Lin *et al*., 2023).

Therefore, the current results highlighted the robust discriminatory capacity of this partial *rpoB* gene sequence among the 15 studied rhizobacterial isolates, which is in accordance with a recent study that has employed these genes for identification of the closely related species (Hashemzadeh *et al*., 2023). This was supported by the concordance in the two phylogenetic trees; one was based on the concatenation of six HKG genes (Fig. 4A) and the other solely on *rpoB* gene (Fig. 4B). Similarly, a previous study conducted by Chouati *et al*., (2023) was unable to distinguish between the two bacterial species without conducting a Multi-Locus Sequence Typing (MLST) using the six genes.

Furthermore, the phylogenetic analysis of the 90 *rpoB* gene sequences (Fig. 5) provided additional supporting evidence for these findings, given that this phylogenetic analysis has been recently documented as an effective method for species discrimination (Tsoukalas *et al*., 2024). This is the first description of *rpoB* gene partial sequence as a phylogenetic marker, which provides a simple and accurate method compared to the previous used phylogenetic and phenotypic approaches, to distinguish *B. paralicheniformis* from the presumptive *B. licheniformis* isolates, or even from the other *Bacillus* isolates identified at the genus level.

Conclusion

In conclusion, our study underscores that relying solely on 16S rRNA sequencing is insufficient for accurate discrimination of *B. paralicheniformis*, given its high similarity to other bacterial species such as *B. licheniformis*. Therefore, the *rpoB* gene partial sequence was used as a reliable method to differentiate *B. paralicheniformis* from its closely related *B. licheniformis*. This approach serves as a rapid and valuable molecular marker, complementing the standard 16S rRNA sequencing method in routine laboratory identification. Our

study identified two highly active and stress-tolerant PGPR *B. paralicheniformis* isolates out of the 15 studied isolates. These isolates displayed tolerance to abiotic stresses and demonstrated significant PGPR properties. Thus, they represent a promising avenue for the development as bio-fertilizers.

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Conflict of interest

The authors declare that no conflicts of interest exist.

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Ethical approval

Non-applicable.

Author's Contributions

Conceptualization, M.A., and M.M.; Roles/Writing - original draft, M.A.; Data curation, M.A. and A.M.; Formal analysis, T.C. and B.R.; Investigation, B.R., and A.M.; Methodology, M.A., A.M. and T.C.; Project administration, E.E. and M.M.; Software, M.A.; Resources, C.T.; Supervision, M.M. and B.R.; Visualization, M.A and C.T.; Validation, B.R., E.E and M.M.

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