Isolation and characterization of plant growth promoting bacteria (PGPB) from the rhizosphere of *Spinacea oleracea L.*

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Abstract

As the years pass by, there is an increase in abiotic stress conditions around the environment that directly or indirectly affect agriculture around the world. Therefore, there is a dire need to increase the sustainability of plants. Plant Growth Promoting Bacteria (PGPB) play an important role in maintaining the physiology and growth of plants under various stress conditions.

This study looks into the isolation and characterization of different PGPB from Spinacia oleracea L. and their tolerance against salinity and commonly used commercial pesticides against the Spinacia family. The techniques used are isolation by serial dilution, 16sRna sequencing, characterization of different PGPB assays for confirmation such as ammonia production, catalase test, phosphate solubilisation, potassium solubilization, siderophore production, indole-3-acetic acid production, biofilm formation assay, halotolerance and tolerance study using Minimal Inhibitory Concentration (MIC). PGPB were isolated and characterized from Spinacia oleracea L., which was under an abiotic stress environment.

Isolates were Bacillus clarus, Bacillus licheniformis, Paenibacillus alvei SJ6 and Paenibacillus alvei SJ8, having quantities as high as 78.1±0.004 mgL^{-1} phosphate solubilization, 43.8 mgL^{-1} of indole-3-acetic acid production, 14.566±0.011 psu of siderophore production and 0.62 ±0.027 µmol mL^{-1} of ammonia production. All isolates also had considerable amounts of halotolerance up to 10%, whereas Bacillus licheniformis had 12.5% halotolerance. The bacterial isolates had considerable tolerance against commonly used commercial pesticides against green leafy vegetables such as chlorpyriphos + cypermethrin combination and fungicides such as mancozeb. Therefore, this study looks into the isolation of potential plant growth promoting bacteria that have considerable amount of halotolerance and pesticide tolerance.

Keywords: Abiotic stress, Plant Growth Promoting Bacteria (PGPB), Pesticide, Halotolerance, Tolerance.

Introduction

Abiotic stresses that are detrimental to plant growth and development include fluctuations in temperature, excess or insufficient water, high salt levels in soil, heavy metals and exposure to UV radiation. These stresses also cause significant global crop output losses. Just 9% of the world's agricultural land is suitable for growing crops and the remaining 91% is subject to stressors that frequently occur in combination[24]. Abiotic stresses account for almost half of agricultural output losses, but as a result of overuse of natural resources and drastic climate change, their severity and negative effects are projected to increase significantly[14]. Since these plants are sessile, they must withstand abiotic challenges like salt, drought and extremely high or low temperatures. These stresses negatively impact the crops in terms of their growth, propagation, as well as productivity. Also, overuse of fertilizer and pesticides has a detrimental effect on the soil quality.

As a result, because 98% of the world's food is produced on land, corrective action is required to stop land degradation[29]. Due to rising desertification brought on by climate change and the degradation of agriculturally marginal areas, the quantity of land that can be exploited for agriculture is still limited. Plant developmental activities including seed germination and the rates of photosynthesis, respiration and transpiration, are impacted by these stresses, which lower plant growth, yield and quality. In order to counteract the detrimental effects of these stressful situations, both biotic and abiotic adaptations to agricultural techniques are therefore necessary[9].

One of the most commonly practiced biotic methods is plant-associated microbiomes. They hold enormous potential for enhancing plant yields and resilience in agricultural settings. An increasing amount of research shows that in contrast to abiotic methods that harm crops and soil health, biological technologies utilizing bacteria or their metabolites can improve nutrient uptake and production, manage pests and lessen plant stress reactions[41]. By a number of different processes, "plant growth-promoting bacteria" (PGPB) can protect plants from environmental stresses and several plant infections.

Several important bacterial traits such as biological nitrogen fixation, phosphate solubilization, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, biofilm formation, HCN production and production of siderophores and phytohormones, can be assessed as plant growth promotion (PGP) traits[38].

Through the secretion of various metabolites and hormones, nitrogen fixation and mineral solubilization, plant growth-promoting bacteria (PGPB) increase the bioavailability of other nutrients and foster soil health. The ability of these bacteria to colonize plant roots, the exudation produced by plant roots and the condition of the soil are some of the elements that affect the efficacy and success of PGPB as inoculants for agricultural crops. The ability of PGPB to colonize roots is directly related to microbial survival and competition in the soil, as well as to the regulation of gene expression and quorum sensing-mediated cell-to-cell communication[15].

One abiotic method applied for crop production improvement is the application of pesticides. Without a corresponding rise in food production, the global population growth of the 20th century would not have been conceivable. The use of pesticides influences the production of about one-third of agricultural products. without which fruit output would have decreased by 78%, vegetable production by 54% and cereal production by 32% if pesticides were not been used. As a result, pesticides are essential for lowering disease rates and raising crop yields across the globe[42]. In agriculture, farmers usually prefer to apply less expensive chemical commercial pesticides to protect their crops. On the other hand, increased pesticide use in agricultural regions contaminates the soil. One possible reason for pesticides' harmful effects on plants could be a decrease in this helpful bacterial group brought on by exposure to the chemicals[1].

On green leafy vegetables, imidacloprid, chlorpyrifos, profenofos, fungicides and cypermethrin are the pesticides most frequently used[4,26]. According to Ramani[32], the impact of chlorpyrifos on phosphorus-dissolving microorganisms revealed a modest suppression of anaerobic PSB in a submerged clay loam. The growth of phosphate-solubilizing bacteria was inhibited by simazine and 2,4-dichlorophenoxyacetic acid, while triflusalin had no effect on the mineralization of phosphorus. Therefore, different PGPBs might have different responses and tolerances to the different pesticides applied. "Hence, the current study aims to isolate and characterize potential PGPB from the rhizosphere of *Spinacea oleracea L.,* as well as their ability to tolerate a commonly available pesticide.

Material and Methods

Experimental Site: The bacteria were isolated from the rhizosphere of *Spinacea oleracea* grown at Aluva State Seed Farm, Kerala, India (10°7'0"N 76°21'40"E). As required by the experiment, this location fulfils the criteria of the study. The soil in this area is highly acidic and the area has extreme hot temperatures[39,44,45].

Isolation, Identification and Characterization of bacteria: The soil samples (in triplicate) were collected from the plant's rhizosphere and preserved at 4 °C in sterile zip-lock plastic bags while adhering to aseptic protocols. It was also labeled with the source and location. The samples that were gathered were transported to the laboratory so that soil bacteria could be isolated using the serial dilution method[2]. Conventional methods, such as morphological analyses, culture characteristics, staining methods and biochemical analyses such as the methyl red test, simmon citrate test, indole test, Voges Proskauer test and catalase test, were used to characterize the isolated bacteria[30]. Characters related to morphology including colour, elevation and colony edge, were also recorded after pure culture isolation. Initially, the gram nature of each isolate was ascertained by staining with crystal violet and safranin as per Capuccino[6].

The isolated bacterial isolates were identified by 16S rRNA gene sequencing[13]. With the use of universal 16S rRNA-F and 16S rRNA-R primers forward (5′-AGAGTTTGA TCMTGGCTCAG-3′) and reverse (5′-CTGCTGCSYCC CGTAG-3′), 16S rRNA gene fragment was amplified and the resulting PCR amplicon was put through forward and reverse sequencing processes. The sequencing was performed at Barcode Biosciences, Bangalore, Karnataka, India. These sequences were analysed using Basic Local Alignment Search Tool (BLAST), analysis and after that, the sequence data was uploaded to the NCBI database in order to receive an accession number. Then Clustal W software was used to pick and align multiple matching sequences for phylogenetic tree construction and Molecular Evolutionary Genetics Analysis 11 (MEGA 11) was used to create the phylogenetic tree and distance matrix.

Biofilm Production and Motility: Plant growth-promoting bacteria that form biofilms (PGPBs) have become popular choices for use in agriculture. Bacteria must successfully colonize a plant, be competent in the rhizosphere and then invade interior tissues in order to have any positive effects on the plant. Bacteria can colonize roots and create biofilms when they are motile, which is a crucial characteristic in the interaction between plants and microorganisms[21]. Therefore, these bacterial isolates were cultured in Tryptone yeast (TY) broth for 48 hours under static conditions at 37° to examine their ability to produce biofilm[28]. Following that, adherent cells were rinsed three times with distilled water, followed by the disposal of the supernatant and the addition of 0.1% crystal violet solution for staining the adhering biomass.

The assay for swarming motility was also conducted using the methodology by Kearns[16]. The swarming motility was determined by stab technique. A single colony was chosen and was stabbed all the way to the bottom of the tube through soft agar medium. Incubate it for 18 hours, or until growth is noticeable, at 37°C.

Phosphate Solubilization: Pikovaskya's agar medium supplemented with 1000 mgL^{-1} of tricalcium phosphate was used to assess the phosphate solubilization behavior of bacteria. Growing on this medium, phosphate-solubilizing bacteria will create a clear zone surrounding the colony as a result of nearby phosphate utilization. The bacterial isolates

were spot-inoculated with 3 µL of a recently generated bacterial culture to assess the microbial ability to solubilize phosphate. For ten to fifteen days, the plates were incubated at 28 °C. The bacterial colonies surrounding transparent zones indicate positive results[36].

Indole-3-Acetic Acid (IAA) production: The role of IAA in modulating crop growth by both crops and PGPB is crucial for sustainable agriculture. Plant reactions to environmental changes are regulated by phytohormones which include auxins such as IAA[7]. In order to identify isolates that produced indole acetic acid, bacterial isolates were inoculated in Luria Bertani broth supplemented with 0, 2.5 and 5 mg/mL of tryptophan. The culture was then cultured for 7 days at 28± 2 °C. The culture was then centrifuged at 10,000 RCF for 30 minutes. 4 mL of Solawaski's reagent was combined with 2 mL of supernatant. The appearance of a pink colour signified the qualitative assessment of Indole acetic acid production. Visible spectrophotometry was performed for the quantitative assessment of the IAA production in which the optical density was measured at the wavelength of 530 nm. Then, an IAA-standard curve was used to estimate the amount of IAA produced[17].

Nitrogen fixation test: Jensen's medium is designed in accordance with Jensen's recommendations and is suggested for the identification and culture of bacteria that fix nitrogen. The ability of PGPB to fix nitrogen is determined using this test. The source of energy used in this test is sucrose. The media's sodium molybdate promotes nitrogen fixation. The media's osmotic balance is preserved by sodium chloride. When calcium is available as chloride or sulfate, nodulation is stimulated. After being isolated on nitrogen-free Jensen's medium[34], nitrogen-fixing bacteria underwent additional testing for various PGPR characteristics.

Ammonia Production: PGPB can fix atmospheric nitrogen and convert it to ammonia[25]. Therefore, the ability of these bacterial isolates to produce ammonia in peptone water was examined. In each tube, 10 mL of peptone water was used to inoculate freshly produced cultures which were then cultured for 48–78 hours at 28 ± 2°C. Each tube was added with 0.5 mL of Nessler's reagent. A positive test result for ammonia production was shown by the colour turning from brown to yellow[22], thereby indicating the PGPB's ability to convert complex nitrogen into ammonia.

Siderophores Production: Plants receive iron from siderophores made by soil PGPB, which also aid in the growth of the plants. The strong affinity siderophores for ferric ions is the basis of the chrome azurol sulfonate (CAS) assay, a general chemical method for siderophore identification[10].

Bacterial strains were employed to determine siderophore production using both qualitative and quantitative approaches. The CAS reagent was made in accordance with Schwyn and Neilands for both techniques. The CAS agar plate method was performed to qualitatively analyze the presence of siderophore detection, after which the siderophore producing isolates were first cultivated in nutrient broth at 37°C for 24 hours. Similarly, after that, the isolates were grown in TY broth and cultures were centrifuged for 10 minutes at 10,000 rpm. Ferric chloride solution and CAS assay solution were added and absorbance was read at 630nm[3]:

$$\text{Siderophore production (psu)} = \left((Ar - As) \div Ar\right) * 100$$

where Ar = absorbance of reference (CAS solution and uninoculated broth) and As = Absorbance of sample (CAS solution and cell free supernatant of sample)

Catalase test: The catalase enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas which is a significant enzyme to protect bacterial cells from oxidative stress[29]. A small number of isolated bacteria were applied to the dried slide using a sterile inoculating loop. A tiny drop of hydrogen peroxide was placed on the bacteria's surface and the presence of effervescence indicated a positive result[11].

Hydrogen cyanide (HCN) production: Hydrogen cyanide is one of the secondary metabolites that the PGPR produces and it is crucial for the biological control of pathogens. The Lorck technique[20] was used to screen each isolate for the formation of hydrogen cyanide(HCN). To describe it briefly, isolates were inoculated into Luria Bertani broth with 4.4 g glycine/L. The top of the tube contained a Whatmann filter paper no. 1 soaked in a solution of 0.5% picric acid and 2% sodium carbonate. These tubes were incubated for four days at 28 ± 2°C. The hue changed from orange to red, indicating the formation of HCN.

Salinity tolerance of the isolated PGPB: Salinity is one of the main issues faced in agriculture. Ionic stress brought on by certain ions in soil salts, such as sodium or chloride, is the result of soil salinity's impact on agriculture. Therefore, the isolate's ability to withstand salinity has to be determined.

The isolates were added to nutrient agar that had different NaCl concentrations of 2.5%, 5%, 7.5%, 10% and 12% w/v. Following that, these plates were incubated for three days at 37°C and bacterial growth was monitored every 24 hours[31].

Pesticide tolerance of the isolated PGPB: Pesticides are used in order to get rid of or manage a range of agricultural pests that can harm livestock and crops and lower farm output. Therefore, the isolates have an ability to withstand the particular concentrations of pesticides. The effect of the most widely used commercial pesticides, namely mancozeb (MNZ), chlopyriphos (CHP) and cypermethrin (CYP) for the cultivation of spinach was determined on the bacterial isolates and their tolerance was tested using the minimal inhibitory concentration (MIC) method. The isolate was

added to nutritional agar (NA) plates containing varying pesticide concentrations.

Various commercial pesticides belonging to the family of organophosphates, pyrethoid and fungicides were used in this medium. After the media was autoclaved and cooled to 50°C, these different concentrations of pesticides were added such as mancozeb (3500 mg L^{-1}), a commonly used commercial fungicide and chlopyriphos + cypermethrin (2500 mg L^{-1}) commercially employed pesticide on green leafy vegetables. After 48 hours of incubation at 37°C, the incubated plates were checked for bacterial growth and loopfuls of the viable isolate from this medium were inoculated into nutrient broth to estimate visible growth after 24 hours incubation[23].

Results and Discussion

Isolation and characterization of rhizosphere bacteria: Ten rhizosphere bacteria with different colony morphologies were extracted from *Spinacia oleracea L*. roots. Four out of the ten morphologically unique rhizosphere bacteria, namely P3, P5, P6 and P8, were characterised for the potential PGPB traits. Through the use of BLAST analysis, the sequence was compared to others in the database and it was discovered that P3 and P5 were *Bacillus clarus* and *Bacillus licheniformis,* whereas P6 and P8 belonged to *Penaebacillus* family. The morphological and biochemical characteristics of these four rhizospheric bacteria were analyzed (Table 1). All the isolates were identified as Gram positive and are all motile.

Multiple alignment program Clustal W was used to pick and align the first 10 sequences based on the maximum identity score. Then software MEGA 11 was used to create the phylogenetic tree for P6 and P8[40] (Fig. 1).

P6 and P8 were identified to be the *Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8*. The accession numbers obtained for *Bacillus clarus*, *Bacillus licheniformis*, *Paenibacillus alvei SJ6 and Paenibacillus alvei SJ8* from the GenBank are PP355449, PP355450, PP355538 and PP355543 respectively (Table 2).

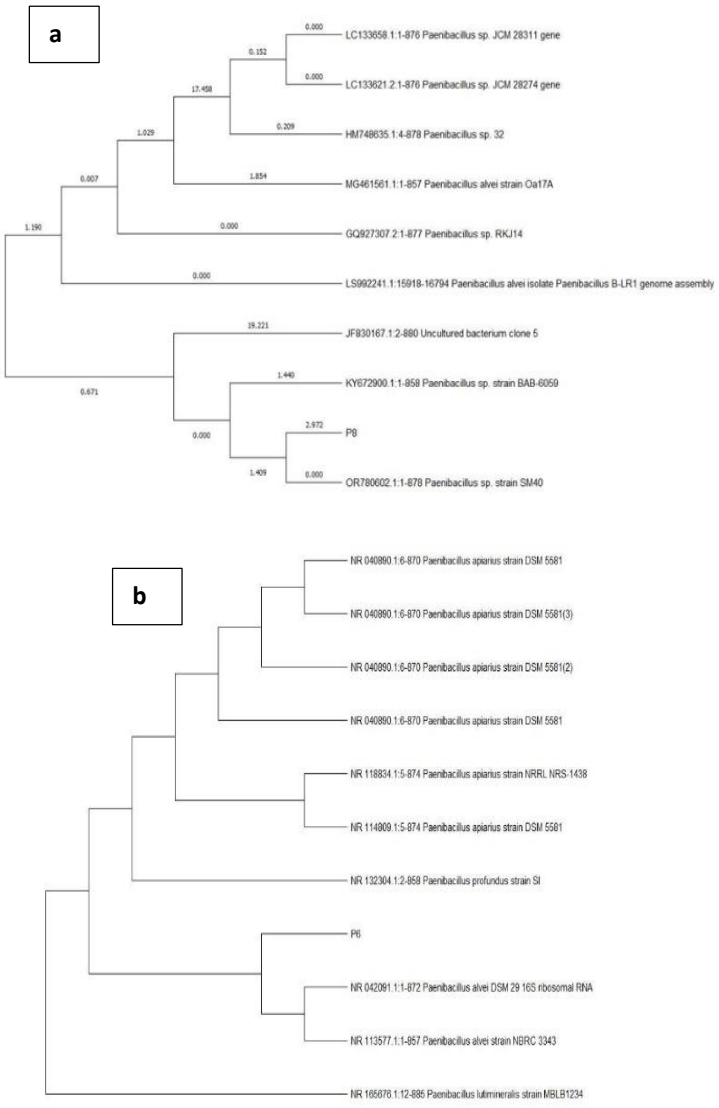

Fig 1: (a) Phylogenetic tree of *Paenibacillus alvei SJ8* and (b) *Paenibacillus alvei SJ6* created using MEGA11.

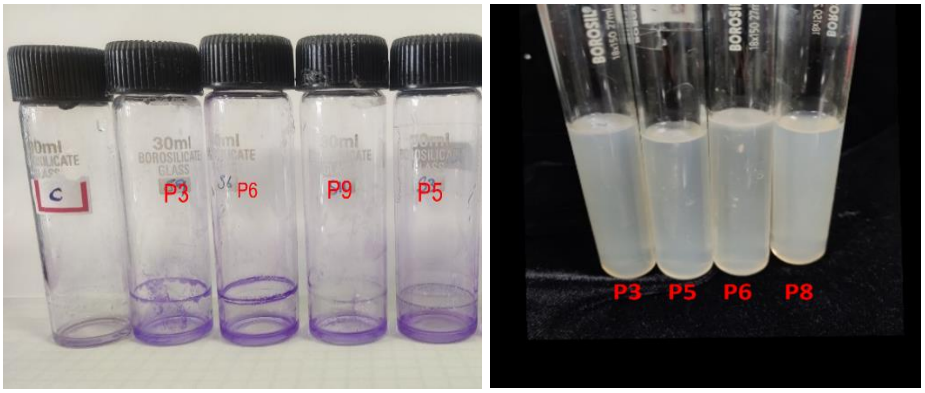

Fig 2: (a) Representation of biofilm formation (b) Motility by stab technique

Table 1
Bacterial characterization: colony morphology and biochemical assay

Bacterial Isolates	Gram Staining	Endospore staining	Motility	Ammonia production	Catalase	Indole	Methyl Red	Voges Proskauer	Simmon Citrate
Bacillus clarus	+	+	+	+	+	+	–	–	–
Bacillus licheniformis	+	+	+	–	+	–	–	+	–
Paenibacillus alvei SJ8	+	+	+	+	+	+	+	–	–
Paenibacillus alvei SJ6	+	–	+	+	+	+	+	–	–

Table 2
Bacterial identification and accession number obtained for the bacterial isolates

Bacterial Isolate Code	Bacteria Identified	Percentage Identity	Accuracy Length	Accession Number
P3	*Bacillus clarus*	98.41%	1552	PP355449
P5	*Bacillus licheniformis*	99.57%	1545	PP355450
P6	*Paenibacillus alvei SJ6*	100%	719	PP355538
P8	*Paenibacillus alvei SJ8*	100%	905	PP355543

Biofilm formation and Swarming Motility: Four isolates, *Bacillus clarus*, *Bacillus licheniformis*, *Paenibacillus alvei SJ6 and Paenibacillus alvei SJ8,* had shown visible biofilm formation due to the appearance of stained rings on the inner walls of respective test tubes (Figure 2a). All isolates had profound swarming motility, as the bacterial growth could be seen along the stab and at the top of the stab point, covering the entire surface (Figure 2b).

Since PGPB can colonize sites, cycle nutrients, boost pathogen defenses and raise tolerance to abiotic stressors, these biofilms can thus raise crop yields and agricultural output[19]. By colonizing the root system and preventing the establishment or suppression of harmful rhizosphere microorganisms, the motility and biofilm formation properties of the strains *Serratia sp.* S119 and *Enterobacter sp*. J49 growing in the presence of peanut, maize and soybean root exudates were found to improve plant growth[21].

Phosphate solubilization assay: The amount of phosphate solubilized in the growth media by the isolated rhizosphere bacteria - *Bacillus clarus*, *Bacillus licheniformis*, *Paenibacillus alvei SJ6 and Paenibacillus alvei SJ8* is 72.9±0.005 mgL^{-1}, 125.5±0.003 mgL^{-1}, 43.6±0.006 $mgL^{-1}$and 78.1±0.004 mgL^{-1} respectively, when supplied with 1000 mgL^{-1} of tricalcium phosphate in the Pikovaskya's media and incubated for 7 days at 37 °C (Figure 3).

In a study, it was seen that two PGPB bacterial isolates assigned HCF6 and HCF9 belonging to the genus *Pantoea* had solubilised phosphate in the phosphate (NBRIP) medium of the National Botanical Research Institute. Both of these isolates[36] effectively solubilized tricalcium phosphate [$Ca_3(PO_4)_2$] by releasing soluble phosphate up to 0.002 mg L^{-1}. In another study, the estimated amount of dissolved phosphate in the NBRIP supernatant ranged from 85 mgL^{-1} to 1312 mgL^{-1} with two isolates, L228 and L132 PGPB, exhibiting the maximum solubilization[27]. Therefore, the amount of phosphate solubilized in the current study by *Bacillus clarus*, *Bacillus licheniformis*, *Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8* had considerable solubilization potential.

Indole-3-acetic (IAA) acid Production: The precursor L-tryptophan (Trp) is the starting point for the Trp-dependent IAA biosynthesis pathway, which produces IAA. Therefore, different concentrations of L-tryptophan were used to identify the optimum production[22]. Without addition of L-tryptophan (0%), bacterial isolates *Bacillus clarus*, *Paenibacillus alvei SJ8*, *Bacillus licheniformis and Paenibacillus alvei SJ6* produced 3±0.0001 mgL^{-1}, 3.6±0.0001 mgL^{-1}, 4.2±0.0001 mgL^{-1} and 6.2±0.0001 mg L^{-1} IAA respectively. After the addition of 2.5% L-tryptophan, they produced 27.3±0. mgL^{-1}, 29.6±0.001 mg L^{-1}, 29.8±0.001 mgL^{-1} and 43.8±0.001 mgL^{-1} IAA respectively. The addition of 5% of L-tryptophan led to the production of 21.5±0.002 mgL^{-1}, 8.73±0.002 mgL^{-1}, 17.06±0.001 mgL^{-1} and 24±0.001 mgL^{-1} IAA respectively (Figure 4).

Therefore, it is seen that when the concentration of tryptophan was increased from 0% to 2.5%, there was a significant increase in IAA production whereas 5% tryptophan did not produce IAA as much as it did in 2.5%. This might be because of increased concentration of tryptophan resulting in the inhibition of enzyme tryptophan-2-monooxygenase (iaaM) and decreased IAA production. Hence the best suited supplied concentration of tryptophan for enhanced IAA production is found to be 2.5%[43].

In a study by Ratnaningsih et al[33], it was found that IAA was produced by six identified PGPB isolates at quantities as high as 36.93 mgL^{-1} *Bacillus sp. NCTB5I* had the highest value followed by *Pseudomonas sp. CHTB 5B* (6.65 mgL^{-1}) and *Brevundimonas sp. CHTB 2C* (13.13 mgL^{-1}). Compared to this study, our isolate, *Paenibacillus alvei SJ6* produced as high as 43.8 mgL^{-1}. In another study, it was found that *Rhizobium sp.* produced the maximum quantity of IAA (90.21 mgL^{-1}), whereas *Phyllobacterium sp.* produced the least amount (67.85 mgL^{-1}). *Bacillus sp.* and *Agrobacterium sp.* produced 83.54 mgL^{-1} and 74.3 $mgL^{-1}$of IAA respectively[18].

Detection of Siderophore production: The production of siderophore was first observed qualitatively on blue agar Chrome Azurol S plate (CAS media) as the blue colour media changed its colour to yellow, indicating a positive result. The traditional method of quantifying was analyzed for the amount of siderophore produced. The bacterial isolates *Bacillus clarus*, *Paenibacillus alvei SJ6*, *Bacillus licheniformis and Paenibacillus alvei SJ8* produced a significant amount of siderophore (Table 3).

In a study, it was observed that when assessed using traditional methods of siderophore production, the concentration of siderophore generated by the bacterial strains ranged from 7.97 ± 0.58 to 69.16 ± 0.71 psu; however, when quantified using the microplate method, it was from 8.33 ± 0.08 to 69.81 ± 0.16 psu. This is due to the sensitivity of the microplate method to read the minute values[3]. Thus, compared to this study, our isolates *Bacillus clarus*, *Bacillus licheniformis*, *Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8* had a considerable amount of siderophore production, with *Bacillus licheniformis* having the highest production of 16.89±0.05 psu.

HCN and Ammonia production: All the isolates were tested for HCN and ammonia production. Several biocontrol PGPB are capable of producing hydrogen cyanide (HCN). The majority of fungal phytopathogens could not proliferate due to the production of biocontrol mechanisms by HCN[25]. Also, it has been demonstrated that the ammonia generated by PGPR provides nitrogen to their host plants, promoting the elongation of their roots and shoots as well as their biomass[5]. To determine the production of HCN after incubation, filter paper soaked in a solution of 0.5% picric acid and 2% sodium carbonate turned yellow to orange-red after incubation, indicating a positive result.

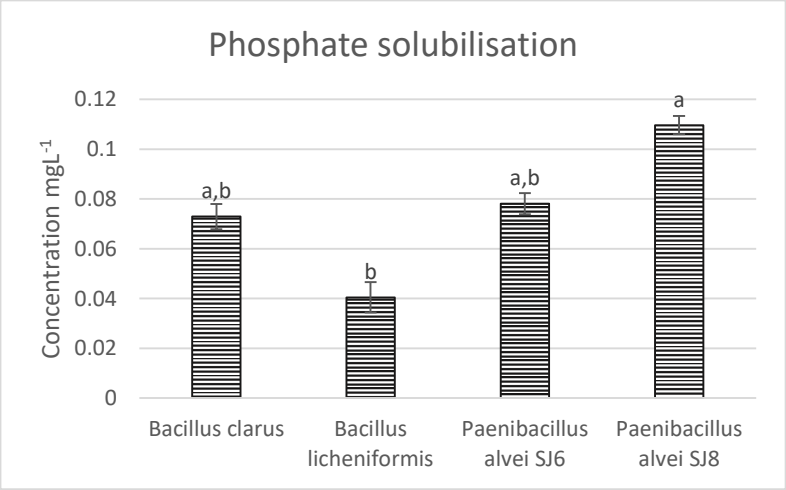

Fig. 3: The bar graphs represent the different concentrations (mgL^{-1}) of phosphate solubilised in the media. Values are average of three replications and the results are shown as mean ± SD. Error bars represent standard deviation. Means followed by the same letter in a column are not significantly different (P = 0.05) by Duncan's multivariate test (DMRT) (P ≤ 0.05).

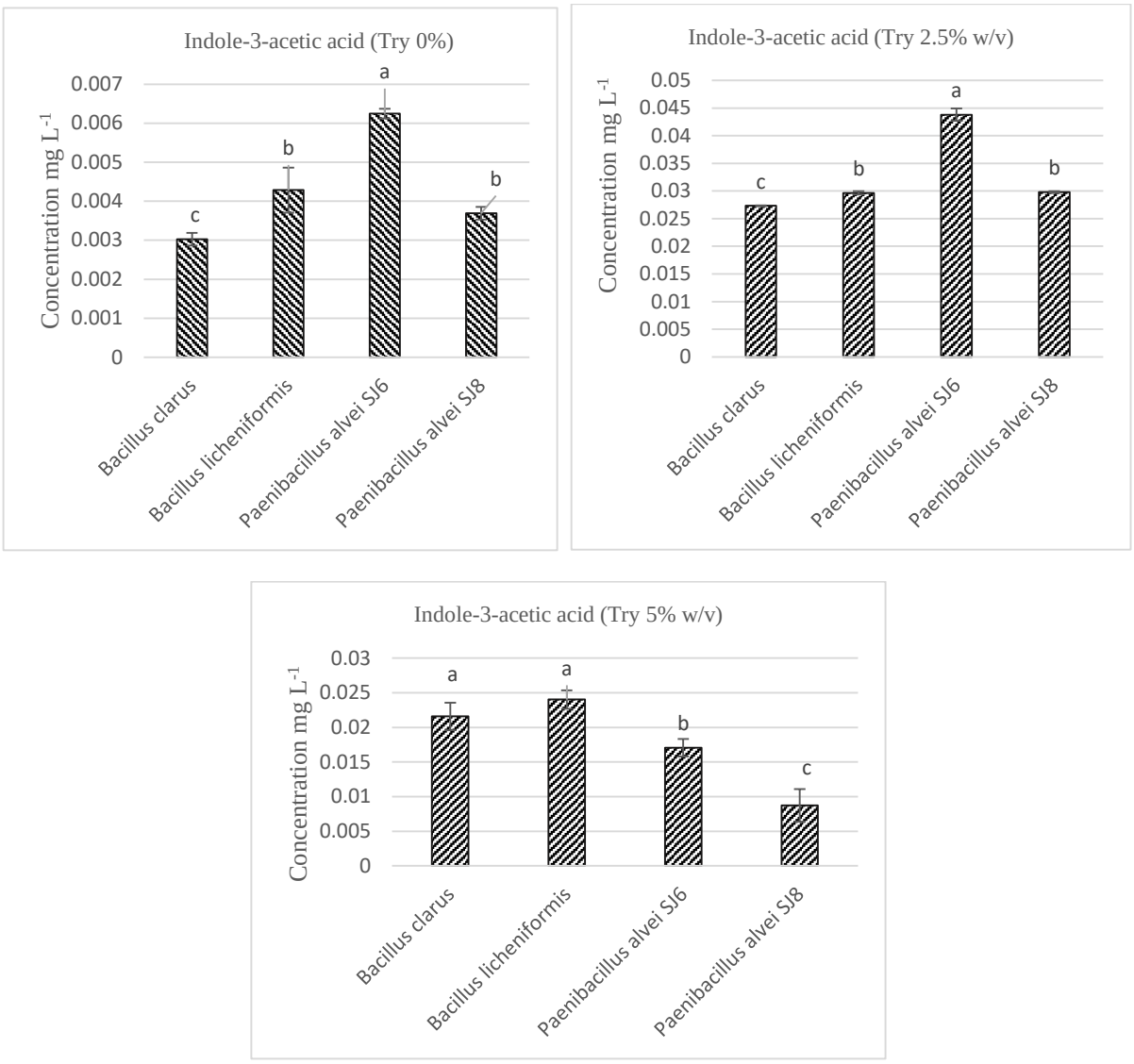

Fig. 4: Production of Indole-3-acetic acid with different concentrations(mgL^{-1}) of L-tryptophan. The bar graphs represent the different concentrations of IAA formed in the media. Values are average of three replications and the results are shown as mean ± SD. Error bars represent standard deviation. Means followed by the same letter in a column are not significantly different (P = 0.05) by Duncan's multivariate test (DMRT) (P ≤ 0.05).

The presence or absence of this color intensity is noted by "+"; intensity of character is designated by the number of "+" signs (figure 5a). All isolates except *Bacillus licheniformis* had significant HCN production.

The ammonia production was detected by the change in colour from yellow to orange when qualitatively assessed using Nesseler's reagent and was detected quantitatively by measuring the absorbance at 420 nm[22]. The amount of ammonia produced by bacterial isolates *Bacillus clarus, Bacillus licheniformis, Penaebacillus alvei SJ6* and *Paenibacillus alvei SJ8* was 0.62 ±0.027 µmol mL^{-1}, 0.601±0.016 µmol mL^{-1}, 0.489±0.016 µmol mL^{-1} and 0.62±0 µmol mL^{-1} respectively (Figure 5b). Thus, these isolates are highly efficient in producing ammonia, among the others in the literature. One of the studies revealed the production of HCN which functions in the substrate as a weathering and metal-complexing agent to increase nutrient availability. HCN has the ability to complex excess micro-elements (such as Fe, Cr, Mo and Co) in soil or to sequester them from compounds like phosphates which are typically combined with these micro-elements to create insoluble precipitates[35]. In another study, it was found that four strains that were studied generated ammonia, albeit at different concentrations: 0.23 to 0.33 µmol·mL^{-1}. The results showed that *B. halotolerans J143* had the lowest value and *E. hormaechei J146* had the highest value[8]. Compared to this, our isolates *Bacillus clarus*, *Bacillus licheniformis*, *Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8* had significant production of ammonia.

Table 4 summarized the plant growth promoting bacterial characteristics such as siderophore production, phosphate solubilization, potassium solubilization, indole acetic-3-acid production, nitrogen fixation, ammonia production, catalase and biofilm production.

Salinity and Pesticide tolerance on PGPB: All isolates except *Paenibacillus alvei SJ8* had significant halotolerance against 10% w/v NaCl salt concentration when incubated for 72 hours at 37°C. *Bacillus licheniformis* had considerable viability in 12.5% NaCl w/v salt concentration as well. This indicates that all isolates except *Paenibacillus alvei SJ8* are significantly halotolerant PGPB (Figure 6).

In one study, the test strain HG-15, belonging to the genus *Bacillus*, showed a high level of tolerance to salt and was able to endure in LB and NA media supplemented with 30% (w/v) NaCl[12]. In another study by Rahman et al[31], 30 isolates met the threshold for halotolerance assay salinity levels up to 20% NaCl, but only seven isolates—six of which were isolated from Ghor Swiemeh were able to tolerate levels up to 25%.

As in the case of pesticide tolerance, all bacterial isolates had considerable tolerance at 2500 mg L^{-1} of a commonly used commercial pesticide for green leafy vegetables (Chlorpyriphos + Cypermethrin). Except for *Paenibacillus alvei SJ6* and *Paenibacillus alvei SJ8,* all isolates also had potential viability on 3500 mg L^{-1}, except for the commonly used commercial fungicide Mancozeb (Figure 7). In a study by Shahid et al[37], it was discovered that rhizobacteria showed differing degrees of pesticide resistance. Strain PS3 of Pseudomonas was able to withstand a notably elevated amount (3200 mg L^{-1}). Furthermore, the degree of tolerance exhibited by isolates of phosphate solubilizing bacteria (PSB) differed according to pesticide species and concentrations.

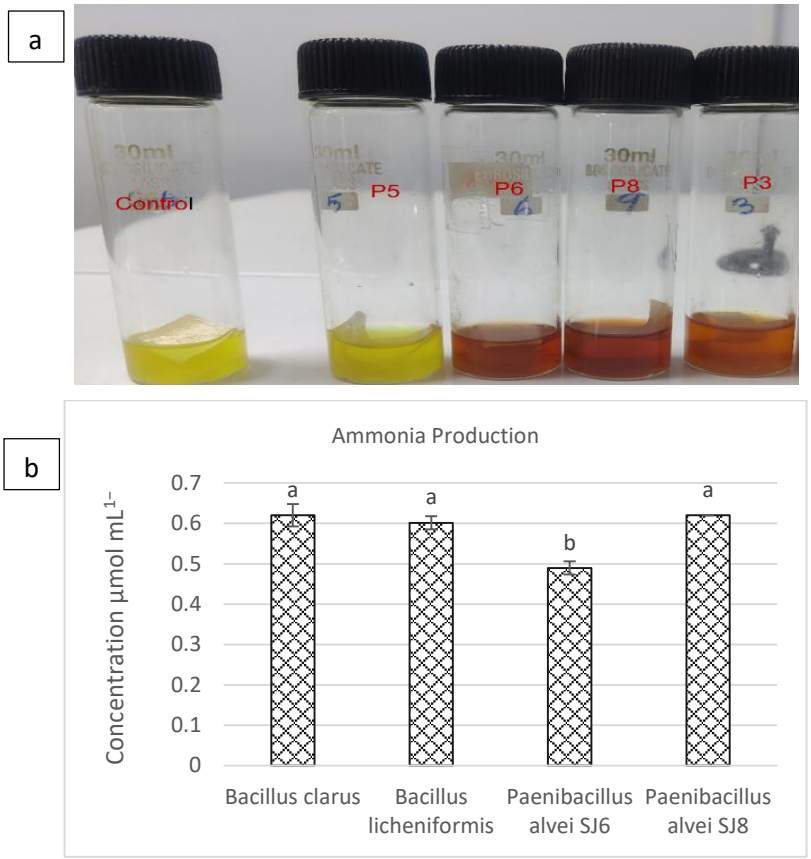

Figure 5: (a) Qualitative representation of HCN production by Locrk's technique. No change in color was reported as a negative response, however the appearance of brick red to light brown was recorded as a strong, moderate, or mild reaction, respectively. (b) Quantitative representation of ammonia production. The bar graphs represent the different concentrations of ammonia produced in the media. Values are average of three replications and the results are shown as mean ± SD. Error bars represent standard deviation. Means followed by the same letter in a column are not significantly different (P = 0.05) by Duncan's multivariate test (DMRT) (P ≤ 0.05).

Table 3
Siderophore production by the bacterial isolates

Name of bacterial strain	Absorbance reference	Absorbance sample	Siderophore production (psu)
Bacillus clarus	0.9963	0.921	7.54±0.026
Bacillus licheniformis	0.9963	0.828	16.89±0.05
Paenibacillus alvei SJ6	0.9963	0.880	11.653±0.037
Paenibacillus alvei SJ8	0.9963	0.851	14.566±0.011

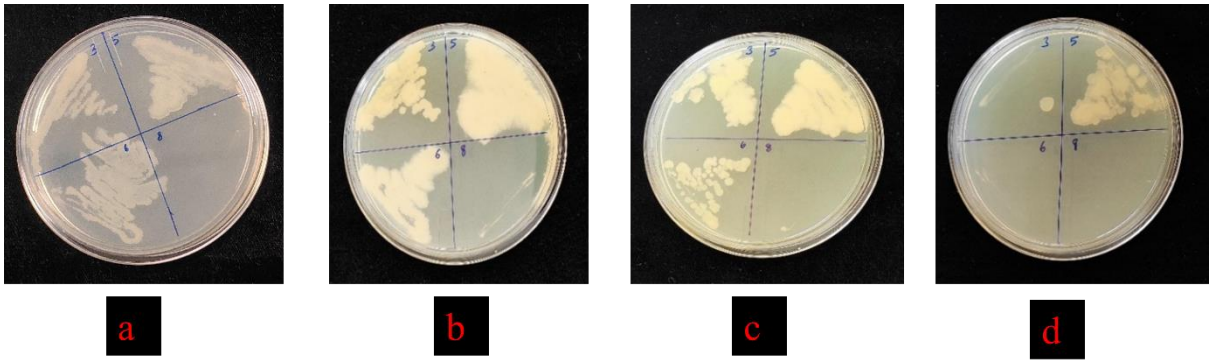

Figure 6: Halotolerance test (a) 5% w/v NaCl (b) 7.5% w/v NaCl (c) 10% w/v NaCl (d) 12.5% w/v NaCl was incubated for 72 hours at 37°C.

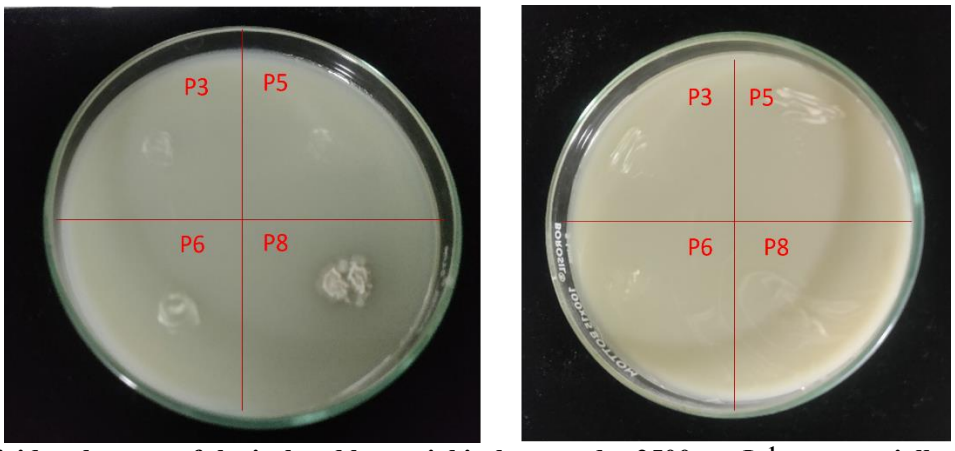

Fig 7: (a)Pesticide tolerance of the isolated bacterial isolates under 2500 mg L^{-1} commercially used pesticide combination (Chlorpyriphos + Cypermethrin) (b) 3500 mgL^{-1} of commercially used fungicide mancozeb.

Table 4
Summary of plant Growth Promoting Bacterial traits of isolated bacterial strains

Bacterial isolate code	Siderophore production	Phosphate solubilisation	Potassium solubilisation	IAA	HCN	Nitrogen fixation	Ammonia production	Catalase	Biofilm formation
Bacillus clarus	+	+	-	++	+	+	++	+	++
Bacillus licheniformis	-	-	-	++	-	+	-	+	-
Paenibacillus alvei SJ6	++	+	-	+	++	+	+	+	++
Paenibacillus alvei SJ8	++	-	-	+	++	+	+	+	++

**presence or absence of character is noted by "+" or "-" sign; intensity of character is designated by the number of "+" sign.

Conclusion

This study presents the successful isolation and characterization of plant growth promoting rhizobacteria from the roots of *Spinacia oleracea L.* surviving in an abiotically stressed environment. The identified isolates *Bacillus clarus, Bacillus licheniformis, Penaebacillus alvei SJ6* and *Paenibacillus alvei SJ8* have shown higher activities of combinations of different PGPB characteristics such as siderophore production, phosphate solubilization, indole-3-acetic acid production, hydrogen cyanide (HCN) production and biofilm formation compared to others from the literature. The results indicate that the isolated bacterial samples *Bacillus clarus, Bacillus licheniformis and Penaebacillus alvei SJ6* have considerable halotolerance up to 10%, except *Paenibacillus alvei SJ8.*

The isolated bacteria also showed potential pesticide tolerance up to 3500 mg L^{-1} of MNZ and 2500 mg L^{-1} (CHP+CYP) pesticide combination except *Penaebacillus alvei SJ6* and *Paenibacillus alvei SJ8*. The obtained results indicate that the isolated rhizobacteria are potential PGPB candidates and can tolerate salt as well as pesticide stresses. Further study has to be conducted to explore its potential and effect on their application on the growth parameters of plants under different stress conditions and further on its application in agriculture.

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