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Isolation, characterization and Formulation Development of Salt Tolerant Plant Growth Promoting Bacteria

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ABSTRACT: Soil salinity poses a global threat to world agriculture by reducing the yield of crops and ultimately the crop productivity in the salt affected areas. Soil inoculation of salt tolerant Plant Growth Promoting Bacteria (ST-PGPB) i.e. free living diazotrophs and phosphate solubilizers not only help in achieving food security but also results in fortifying macro and micro nutrients for mankind and quality of soil organic nutrients, improvement of agro-ecosystem with minimum pollution hazards. Three salt tolerant native PGPB were isolated from Vaso farm and screened based on their abilities to fix N and solubilize P. Isolates AAU V2 and AAU V8 were found N-fixer while, AAU V1 was found P-solubilizer based on their growth characteristics on selective media. The salt tolerance (i.e. NaCl and KCl) test showed that all three isolates were capable to grow at varied salt concentration i.e. from 4.5 to 8.5 % *in vitro*. A liquid assay test on survival and multiplication ability of chosen isolates indicated that all three isolates grows well in all tested growth medium except minimal salt broth without carbon source. ST-PGPB isolates were identified by 16S *r*RNA gene sequencing technique as *Priesta* (= *Bacillus*) AAU V1 nr. sp. *flexia* (NCBI ACC. No. OR287447), *Cupriavidus* AAU V2 nr. sp. *alkaliphilus* (NCBI ACC. No. OR287481) and *Lysobacter* AAU V8 nr. sp. *yangpyeongensis* (NCBI ACC. No. OR287436). The isolates were further tested for PGP traits and found that all three isolates were positive for the availability of macro nutrient (N, P and K). The highest nitrogen fixation (20.25 mg N g^{-1} of sucrose consumed) was recorded by *Cupriavidus* AAU V2, followed by *Lysobacter* AAU V8 (18.70 mg N g^{-1} of sucrose consumed) and *Priestia* AAU V1 (8.05 mg N g^{-1} of sucrose consumed). Maximum P solubilization zone was recorded for isolate *Priestia* AAU V1 (zone of 10.2 mm). All three isolates found moderate K solubilizer and good zinc solubilizer. Moreover, found to produce IAA and ACC deaminase enzyme. A liquid biofertilizer formulation, Bio NP consortium was prepared by mixing all three chosen isolates in equal proportions and further evaluated for its shelf life study up to one year. Shelf life study showed that survival of bacteria in consortium was found excellent up to 6 months and minimum count sustained as per FCO up to 1 year.

Keywords: Plant growth promoting bacteria, PGP traits, Bio NP formulation, Salt tolerant, N fixation, P solubilization, Shelf-life.

INTRODUCTION

The worldwide enhancement in human inhabitants and the associated environmental degradation has largely influenced global food sector due to agro-ecological pollutions to produce adequate food to nourish the world population (Nagangoudar *et al.*, 2023; Glick, 2012). The global population is estimated to be approximately 7.8 billion and anticipated to rise up to 9.7 billion by 2050 (Annonymous, 2017) and generated huge demand for food and feed (Mesa-Marín *et al.*, 2019). However, crop production per unit of land cultivated and growth rate is not enough to meet the desired world demand for 10 billion people in 2050 (GAP, 2018). Due to human interventions and pressure on environment special efforts are needed to raise agricultural productivity.

Soil salinization is a threat for crop production and the quality (Shrivastava and Kumar 2015). It is believed that about 20 percent of the world's irrigated land is affected by gradually increasing salinity (Kumar and Sharma 2020). The plant growth promoting bacteria, which can withstand elevated salt concentration, can improve plant growth by enabling resource use or moderating plant hormone levels or indirectly by reducing the adverse effects of various biotic factors. The use of PGPB is a promising agricultural approach for salinity susceptible crops to tolerant and maintains an appropriate level of productivity (Nadeem *et al.*, 2012; Singh *et al.*, 2011) and may be considered as a promising tool of integrated management system.

PGPB promotes plant growth by number of mechanisms (Bajeli *et al.*, 2023) which includes improvement of macro and micro plant nutrient uptake, regulation and production of various phytohormones,

nitrogen fixation, siderophore production, induced systemic resistance (ISR) etc. (Basu *et al*., 2021; Zahir *et al*., 2001; Kloepper *et al*., 1980). ST-PGPB helps plant to grow better and growth promotion, increases nutrient uptake and antioxidant enzymes to tolerate high salt. Since ST-PGPB have the ability to survive in osmotic and ionic stress conditions, they can be exploited for reclamation of salt affected soils simultaneously to harvest best yield in saline soils (Saiyad *et al*., 2023; Egamberdieva *et al*., 2019; Chaudhary *et al*., 2023).

Detailed information about native bacterial population, their characterization and identification are needed to understand the diversity of indigenous bacteria in the moderate saline soil. Region-specific microbial inoculants can be utilized as bio-inoculum to achieve desirable crop production (Deepa *et al*., 2010). Taking into consideration the above challenging issues, the present research has been designed.

MATERIAL AND METHODS

Isolation of native salt tolerant PGPB. Total 15 soil samples were collected from different locations *viz*. Agronomy farm, Horticulture farm and Nursery; from Vaso campus farm (22°67′N, 72°77′E) for isolation of native salt tolerant PGPB. Samples were collected via standard procedure soil sample were serial diluted up to 10^{-9} dilutions and aliquots (0.1 ml) from each dilution were spreaded on respective selective medium such as modified Jensen's media and modified NFB media for growth of nitrogen fixing bacteria and modified Sperber's media for P solubilizers (Patel *et al*., 2022; Prajapati *et al*., 2016). Inoculated plates were incubated at 30 ± 2˚C for 3-5 days and observed regularly for growth nitrogen fixers and for zone of P solubilization. Selected colonies were pure cultured and stored at 4˚C for further studies.

Screening of native PGPB for salt tolerance. The selected isolates were allowed to grow in nutrient broth having presence of higher salts concentrations of NaCl and KCl. Nutrient broth prepared in saline water collected from Vaso tube well (EC > 4.0 dsm^{-1}) supplemented with dextrose having different NaCl concentrations (4.5, 5.5, 6.5, 7.5 and 8.5 %) and KCl concentrations (4.5, 5.5, 6.5, 7.5 and 8.5 %) were prepared and autoclaved (Kyaw *et al*., 2022). The broth with regular NaCl and KCl level i.e. 0.5 %; was used as control. Inoculation of three cultures of bacteria was carried out in each broth tube followed by incubation at 35 ± 2°C temperatures for 24 hours. The broth tubes were observed for turbidity.

Liquid assay test for PGPB isolates. The nutrient broth tubes having different growth conditions (Yavarian *et al*., 2022) such as 1% Glucose broth, Mineral salt broth and respective broth for nitrogen fixer and phosphate solubilizer were prepared in saline water (pH – 8.2) collected from Vaso campus farm. Five treatments consisted of (T_1: Saline water + 1 % Glucose; T_2: Saline water + Minimal salts; T_3: Saline water + 1 % Peptone; T_4: Glucose Phosphate broth in saline water and T_5: Respective growth medium prepared in saline water i.e. NFB and Sperber's medium) were formulated and 4 repetitions of each treatment were inoculated with respective isolates having 10^7 viable counts. The optical density (OD) values were recorded everyday up to 7 days for growth of isolates in saline condition.

Characterization of salt-tolerant bacterial isolates for plant growth-promoting activity. The isolates were further screened for PGP activities such as nitrogen fixation, phosphate solubilization, potash solubilization, zinc solubilization and production of indole acetic acid (Dhole *et al*., 2023; Chaudhary *et al*., 2023).

Nitrogen fixation. Quantitative estimation of nitrogen was determined by Micro-Kjeldahl method (Kjeldahl, 1983). Five ml of culture was withdrawn and digested with 5 ml of concentrated sulphuric acid and 5 g of digestion catalyst (K_2SO_4 and $CuSO_4$ in 10:1 ratio) until the content turns colorless. After sufficient cooling, the aliquot was transferred to the Micro-kjeldahl distillation unit. Ten ml of 40 per cent sodium hydroxide was added and steam distilled. Ammonia evolved was collected in 20 ml of two per cent boric acid containing two drops of double indicator (83.3 mg bromocresol green + 16.6 mg methyl red indicator dissolved in 10 ml of 95 per cent ethanol) and back titrated against N/50 H_2SO_4. The rate of nitrogen fixation was expressed as mg nitrogen fixed $gram^{-1}$ of sugar consumed.

Phosphate solubilization. Phosphate solubilization activity of isolates were inoculated by using toothpick on respective Sperber agar plates under aseptic condition and were incubated at 30 ± 2°C for five days with observation on colony diameter and solubilization of TCP every 24 h (Pandey *et al*., 2017).

Potash solubilization. All the isolates were spot inoculated on Aleksandrov agar containing mica for testing potash solubilization (Feng *et al*., 2020). Plates were then incubated at 30 ± 2°C for five days with observation on colony diameter every 24 h.

Zinc solubilization. The solubilization potential for zinc was assessed by plate assay technique. The isolates were inoculated into agar medium containing 0.1% insoluble zinc compounds *viz*., ZnO, ZnS and $ZnCO_3$ incubated at 30º C for 48 hours. The diameters of the clearing zones around the colonies were measured for zinc solubilization ability (Costerousse *et al*., 2017; Kandoriya *et al*., 2023).

Indole acetic acid (IAA) production. All three isolates were grown in glucose phosphate broth containing L-tryptophan (0.005M) for 3 days at 30 ± 2°C on shaker at 100 rpm and then centrifuged at 3000 rpm for 20 min. One ml (1:2) supernatant was mixed with 2 ml of Salkowaski's reagent (Gang *et al*., 2019). The intensity of pink color developed post inoculation indicated IAA production.

ACC deaminase production. Chosen cultures were spot inoculated on Petri plates containing minimal medium supplemented with 3 mM ACC substrate (Gupta and Pandey 2019). Plates containing minimal medium without ACC were as negative control and with $(NH_4)_2SO_4$ (2.0 gm/l) as a nitrogen source served as positive control. The plates were incubated for 3

days at 28 ± 2°C. Growth of isolates on ACC supplemented plates was compared with positive and negative control plates.

Formulation development and shelf life study. Three native ST-PGPB cultures; V1, V2 and V8 were used for development of a combined liquid biofertilizer formulation named 'Bio NP consortium'. *In vitro* plate bioassay was carried out to determine compatibility of selected 3 native ST-PGPB isolates. Each bacterial isolate was grown in NMS broth for 3 days and observed every 24 h for inhibition of the test cultures if any. All the isolates were further cross streaked on NA plates to check out their compatibility at intersect. Longevity of the consortium product monitored through determination by colony forming units (cfu) of bacterial population in the product at monthly interval up to 1 year. A serial dilution method was followed to estimate the bacterial population (cfu/ml) from the formulated consortium (Manva *et al*., 2019).

Molecular characterization of bacterial isolates

Isolation of genomic DNA. Bacterial isolates were inoculated in 5 ml Luria broth and incubated at 30 ± 2°C for 24 h on environmental shaker at 100 rpm. The bacterial cultures (2 ml) were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellets were used for extraction of DNA. The bacterial cell pellet was suspended in 2 ml Tris-EDTA (pH-8.0), 250 µl SDS (10 % w/v) and 10 µl of Proteinase K solution (20 mg ml^{-1}) and incubated at 37°C for 1 h. Subsequently, 5 M NaCl (500 µl) followed by 100 µl CTAB (10 % solution in 0.7 M NaCl) was added and incubated in water bath at 650 C for 10 min. The solution was spun at 8,000 rpm for 10 min after mixing with equal volume of chloroform: isoamyl alcohol (24:1) and upper phase was transferred in to clean eppendorf tube. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well by inverting, spun for 10 min at 10,000 rpm and upper aqueous phase was transferred again to a clean eppendorf tube. From the collected supernatant, the DNA was precipitated with double volume of chilled absolute ethanol. Tube was centrifuged for 10 min at 11,000 rpm, supernatant was discarded and pellet was washed in 70 % ethanol and again spun for 5 min at 11,000 rpm. Ethanol was discarded and pellet was air dried. DNA was re-suspended in 200 µl TE buffer and stored at -20°C till further use (Patel *et al*., 2018).

Amplification and sequencing of 16S *r*RNA. Universal primers were utilized for the amplification of the 16S *r*RNA sequence of the native salt-tolerant PGPB isolates. The amplified product was purified and sequenced from Eurofins Genomics India Pvt. Ltd, Bengaluru. The sequences obtained were analyzed and identified using BLAST search and were compared against bacterial 16S *r*RNA sequence available on NCBI database. The 16S *r*RNA gene sequences were assembled using MEGA 4 software, compared with other strains using NCBI BLAST analysis for identification. The output sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis to identify the cultures and to determine the nearest match by constructing phylogenetic tree using standard method (Patel *et al*., 2018).

RESULTS AND DISCUSSION

Isolation and screening of native salt tolerant PGPB. For the isolation of ST-PGPB in present research, the native saline soil samples were collected from different locations of Agriculture college farm, AAU, Vaso; Dist. Kheda, which lies in 22°67′N, 72°77′E. The soil of Vaso college farm is sandy loam with pH 8.2, EC 0.40 dsm^{-1}. The samples were further used for isolation of total bacteria on NA medium, subsequently nitrogen fixing and phosphorous solubilizing bacteria by growing in standard selective growth media, respectively to get pure cultures. Total 15 bacterial isolates were obtained from three different farm locations of Vaso campus.

Out of 15 samples, 3 potential isolates *viz.,* V1, V2 and V8; were chosen based on their morphological and colony characteristics on plates as well as microscopic examination. They were further screened for their salt tolerance ability, survival and multiplication ability in saline water and their PGP traits.

Salt tolerance test for isolates. The results on salt tolerance test indicated that all three isolates were grown well on all the test salt concentrations i.e. NaCl and KCl concentrations (Yashaswini *et al*., 2023). It was also noted that microbial growth was constant up to 6.5 % (in both NaCl and KCl) and then the value of turbidity value reduces slowly. It was further noted that chosen isolates showed growth up to 8.5 % salt concentration in both the test salt mediums.

Liquid assay test of PGPB in saline water. A liquid assay (with saline water collected from Vaso farm – TDS >3200) was performed to evaluate survival and multiplication ability of chosen ST-PGPB isolates *in vitro.* The results revealed that all three chosen isolates showed best results while growing in 1% Glucose, Glucose phosphate broth and their respective growth mediums (Fig. 1). It was also noted that growth of isolates only in minimal salt broth has least population followed by 1% peptone.

Similarly, Sharma *et al*. (2015) isolated 95 bacterial strains and amongst them, 55 showed plant growth promoting characteristics and salt-tolerance to more than 4% NaCl. Kothari *et al*. (2013) isolated *B. safensis* VK which showed salt tolerance up to 14% NaCl and pH ranging from 4 to 8. Zhang *et al*. (2018) isolated 305 bacterial strains, and amongst them, 162 were tested for salt tolerance up to 150 g/l NaCl concentration. Phylogenic analysis of 74 of these salt-tolerant strains showed that they belong to orders *Bacillales* (72%), *Actinomycetales* (22%), *Rhizobiales* (1%) and *Oceanospirillales* (4%). Upadhyay *et al*. (2009) found that most of the isolates were able to tolerate up to 8% NaCl and belong to the genus *Bacillus*.

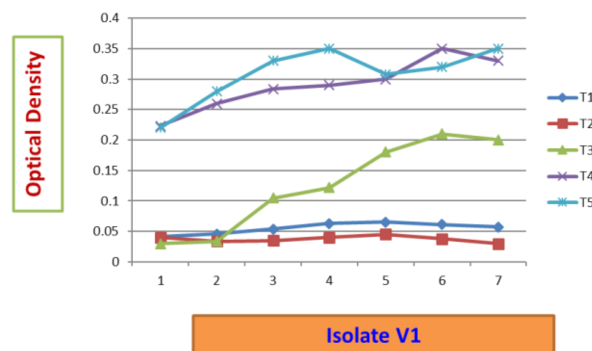

Fig. 1 (A). Survival and multiplication ability of isolate V1 in saline water.

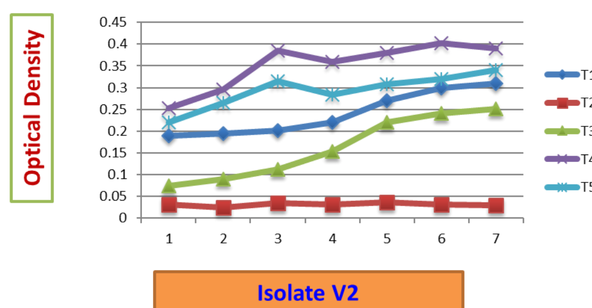

Fig. 1 (B). Survival and multiplication ability of isolate V2 in saline water.

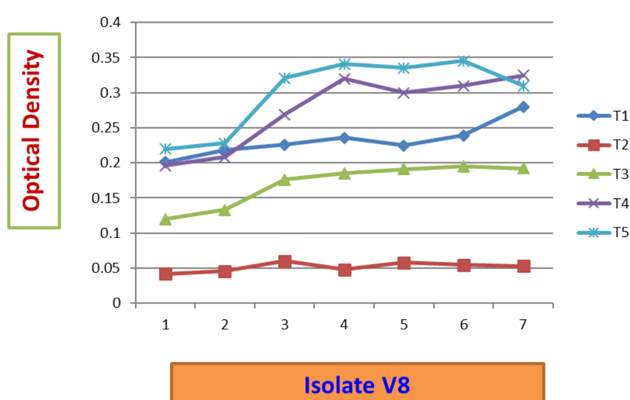

Fig. 1 (C). Survival and multiplication ability of isolate V8 in saline water.

Molecular characterization

Isolation and identification of chosen isolates. To confirm identity of the ST-PGPB the genomic DNA of isolates was extracted, purified, amplified and sequenced from selected isolates. PCR amplification of 16S *r*RNA gene from chosen isolates was carried out using universal primers (U27f and U1492r). 16S *r*RNA partial gene sequence of ~ 1500 bp was carried out (with technical support of Eurofins Genomics India Pvt. Ltd., Bangalore) for sequencing and the output data were stored in FASTA format as described below. The output sequences were subjected for BLAST (Basic Local Alignment Search Tool) analysis to identify the isolates and to find out the nearest match of three isolates (http://www.ncbi.nlm.nih.gov). Identification of isolates based on 16S *r*RNA sequencing is presented in Table 1 and Phylogenetic tree of all three chosen isolates is displayed in Fig. 2(a), (b), (c), respectively.

Table 1: Identification of chosen isolates by 16s *r*RNA gene sequencing.

Isolate	Most closely related organisms*			
	Accession description	GenBank® ACC. No	% Gene identity	% Query coverage
Priesta sp. (=*Bacillus*) AAU V1	NR_113800.1	OR287447	90.52	100
Cupriavidus sp. AAU V2	NR_102851.1	OR287481	99.85	99
Lysobacter sp. AAU V8	NR_043625.1	OR287436	97.44	99

The different studies on native salt tolerant bacteria characterization have been confirmed as *Priesta* (= *Bacillus*) AAU V1 nr. sp. *Flexia* (P-solubilizer), *Cupriavidus* AAU V2 nr. sp. *Alkaliphilus* (N-fixer) and *Lysobacter* AAU V8 nr. sp. *Yangpyeongensis* (free living N_2-fixer) and all three belonging to agriculturally beneficial genera.

Similar type of finding and salt tolerant microorganisms were isolated and identified by several workers worldwide. Pal *et al.* (2014) reported that bacterial

strain *B. flexus* NM25 (HQ875778) can tolerate saline growth condition and grows well up to 20% (w/v) NaCl concentration and the pH range of 4-12. Yan *et al.* (2018) isolated salt tolerant *L. rhizophilus* sp. nov. which grows well in more than 2% NaCl concentration. Jahn *et al.* (2021) investigated effect on reallocation of protein activity of *C. necator* when grown on different limiting substrates and found strong expression of CBB cycle genes i.e. Rubisco upon growth on fructose.

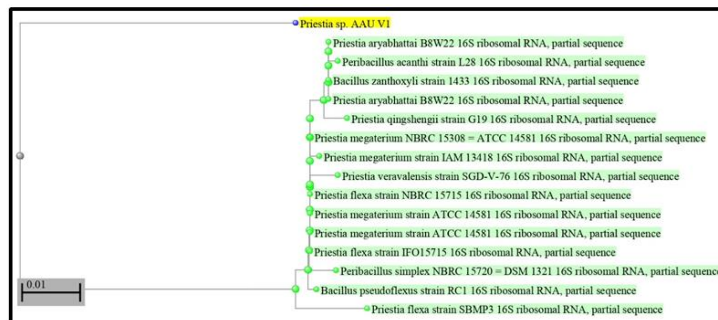

Fig. 2 (a). Phylogenetic tree of isolate AAU V1 *Priesta* sp. (=*Bacillus*)

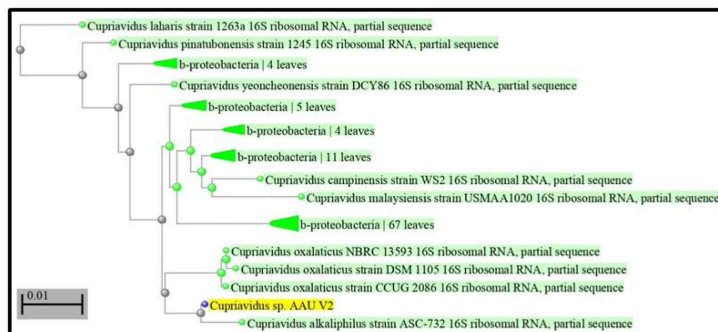

Fig. 2 (b). Phylogenetic tree of isolate AAU V2 *Cupriavidus* sp.

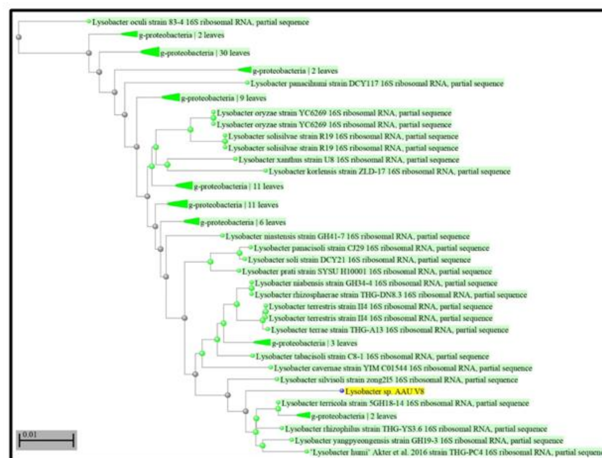

Fig. 2 (c). Phylogenetic tree of isolate AAU V8 *Lysobacter* sp.

Plant growth promotion trait characterization

Nitrogen fixation. All the isolates were confirmed to have ability of fixing atmospheric nitrogen. It was revealed from the results (Table 2) that nitrogen fixing potentiality of chosen isolates ranged from 8.05 to 20.25 mg Ng^{-1} of sucrose consumed and isolate *Cupriavidus* AAU V2 showing the highest nitrogen fixation capacity among all the isolates (20.25 mg Ng^{-1} of sucrose consumed) followed by *Lysobacter* AAU V8 (18.70 mg N g^{-1} of sucrose consumed).

Table 2: PGP traits of chosen isolates (N fixation, P solubilization and K solubilization).

Isolate	N fixation	P solubilisation		K solubilization	
	mg N g^{-1} sucrose consumed	TCP solubilisation zone (mm)	Colony diameter (mm)	Solubilisation zone (mm)	Colony diameter (mm)
Priestia AAU V1	8.05	10.2	0.6	4.2	0.6
Cupriavidus AAU V2	20.25	4.1	0.4	3.1	0.4
Lysobacter AAU V8	18.70	5.8	0.5	2.8	0.5

Phosphate solubilization. The results indicated that all three chosen isolates were able to solubilize tri- calcium phosphate (Selvi and Thangaraj 2023) in more or less quantity. The isolate *Priestia* AAU V1 formed a clear zone of 10.2 mm diameter on Sperber agar plate (Table 2) while, isolate AAU V8 having a clear zone of 5.8 mm diameter.

Potash solubilization. All three chosen isolates were tested on Aleksandrov agar media (Arati *et al*., 2023) containing mica as natural 'K' substrates and showed zone of potash solubilization but the zone was found inferior compared to the standard KMB (Potash solubilizing and mobilizing bacteria) and as per FCO standard (zone < 5 mm and SI >2) (Table 2).

Zinc solubilization. The results on plate assay technique (Arati *et al*., 2023) indicated that isolates *Cupriavidus* AAU V2 and *Lysobacter* AAU V8 showed significant zone around colonies and justifies their ability to solubilize zinc compounds.

IAA production. Results indicated that all three isolates are capable of producing IAA but, the production of Indole Acetic Acid was low and varied with the isolates. These results showed that chosen isolates have capacity to improve plant growth (Selvi and Thangaraj 2023).

ACC deaminase activity. Among the three chosen isolates *Cupriavidus* AAU V2 and *Lysobacter* AAU V8 were grew well on plates containing ACC as sole nitrogen source (Kasim *et al*., 2016) and confirmed the production of ACC deaminase enzyme.

Overall, PGP traits characterization of chosen isolates showed that for 6 key tests assessed for macronutrients and micronutrients supplementation found positive as summarized in Table 3. PGPB has various salinity resistance strategies and mechanisms of plant growth improvement under salt stress. The ability of PGPB to improve crop yields during salt stress includes many direct and indirect mechanisms such as ferrous iron minerals and inorganic phosphate solubilization, exopolysaccharides and biofilms synthesis, production of phytohormones (Naz *et al*., 2009), increased ACC (1-aminocyclopropane-1-carboxylate) deaminase activity (Bal *et al*., 2013) and nitrogen fixation (Sadiki and Rabih 2001).

Table 3: Overall PGP traits of chosen isolates.

Sr. No.	PGP trait	Isolate V1	Isolate V2	IsolateV8
1.	N fixation	+	+++	+++
2.	P solubilization	+++	+	+
3.	K solubilization	+	+	+
4.	ACC deaminase production	+	++	+
5.	Zinc solubilization	+	++	++
6.	IAA production	+	+	+

Note: +++ strong, ++ moderate, - absent

ST-PGPB Bio NP consortium formulation and its shelf life study. Bio NP consortium was formulated by mixing all three isolates in equal proportions (having microbial population count 1×10^9cfu/ml each) at room temperature in laboratory. The data pertaining to shelf life (Singh *et al*., 2022) of microbial population (Fig. 3) revealed that, the bacterial population was maintained well above 10^9cfu/ml up to 270 days. It was also noted that even after 270 days of consortium formulation, the total count remained good and permissible limit of FCO at room temperature.

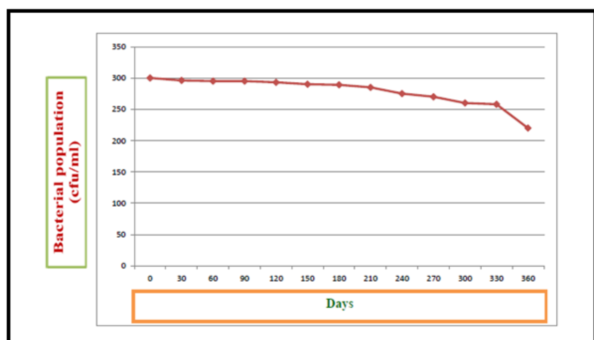

Fig. 3. Shelf life study of Bio NP consortium.

CONCLUSIONS

The present investigation was carried out to isolate and characterize native salt tolerant PGPB from low saline soil of Vaso farm. Three potential isolates were selected based on their morphological characters, salt tolerance abilities and abilities to fix atmospheric nitrogen and soliubilize phosphorous. Morphological and molecular characterization identified the native salt tolerant isolates as *Priesta* (= *Bacillus*) AAU V1 nr. sp. *Flexia* (NCBI Accession no. OR287447) as P solubilizer; *Cupriavidus* AAU V2 nr. sp. *Alkaliphilus* (OR287481) as free-living N-fixer and *Lysobacter* AAU V8 nr. sp. *Yangpyeongensis* (OR287436) as free-living N-fixer. Overall, PGP traits showed that for 5 key tests assessed for macronutrients the isolates found better and good for Zinc, ACC deaminase and IAA production. On the whole, ST-PGPB isolated from low saline soil of Vaso region exhibited good salt tolerance and best plant growth promoting traits in laboratory. The native Bio-NP bacterial liquid consortium product prepared was sustained well at ambient temperature in laboratory.

FUTURE SCOPE

Enhanced production with improved soil fertility in an eco-friendly manner is the need of hour. Isolation and identification of ST-PGPB from saline soil needs to be done at regional and global level. PGP traits characterization and molecular characterization of native ST-PGPB is required for sustainable agriculture system.

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Conflict of Interest. None.

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