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Plant growth efficiency of salt tolerant bacillus sp co inoculation with rhizobium sp

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Abstract
Soil salinity has emerged as a serious issue for global food security. Salt tolerant bacteria were isolated from paddy filed and its efficiency on salt stress were evaluated by in situ method. The conventional methods were used to isolate and characterization of bacteria. *Ability of isolate* thrives in extremely salt concentrations is first evaluated in this study. Isolate *positive* sp showed positive response on salt tolerance, siderophore production, phosphate solubilisation and chromium reduction. Seed germination results confirmed the beneficial effects of *Bacillus* sp and *Rhizobium* sp individually with lowest vigour index. Plant growth promoting efficiency among dual culture showed significant results in root length, shoot length and germination rate, when compared to uninoculation, co inoculation showed significant seed germination index. The present research concludes co culturing of strain *Bacillus* sp with Rhizobium sp has the highest capability for stimulating plant under 3% of salt stress. PGPR mediated mechanisms of salt tolerance in tested plant and future research trends of using ST-PGPR for reclamation of saline soils.

Keywords: auxin, siderophore, co inoculation, salt stress, rhizobium

Introduction
Salinity is a major threat to modern agriculture causing inhibition and impairment of crop growth and development. Worldwide, 800 million hectares of soil is affected by salinity. Salinity is considered the scourge of intensive agriculture. The costs, associated with soil salinity are potentially enormous, and the effects of salinity may impact heavily on agriculture, biodiversity and the environment (Paul and Nair, 2008) [14]. Plant responses to salinity have been divided into two main phases. An ion- independent growth reduction, which takes place within minutes to days, causes stomata closure and inhibition of cell expansion mainly in the shoot (Rajendran *et al*., 2009) [15]. Na^+ and K^+ homeostasis plays a vital role in the growth and development of higher plants under salt conditions owing to potassium– sodium (K^+–Na^+) interaction and is often associated with K^+ deficiency. Salt stress affects photosynthesis both in the short and long term. In the short term, salinity can affect photosynthesis by stomatal limitations, leading to a decrease in carbon assimilation (Parida and Das, 2005) [13]. This effect can produce rapid growth cessation, even after just a few hours of salt exposure. Despite their importance as signaling molecules regulating cellular responses to various stresses (Apel and Hirt, 2004) [4], ROS can also damage plant tissues during salinity stress by perturbing enzyme, cell wall and membrane function. Bacteria that grow in the absence of salt and in the presence of high salt concentrations are known as halotolerant. Non-halotolerant which can grow in low salt concentration about 1% w/v. Slightly tolerant as *Pseudomonads*, *Enterobacteria*, and *Vibrios*, can survive in up to 2–8%, moderately tolerant 18–20% and extremely tolerant microbes can grow over the whole range of salt concentrations from zero to saturation (Flowers and Colmer, 2015) [10]. Salt tolerance and dependence are the characteristics of some microbes. Salt-tolerant microbes can survive in osmotic and ionic stress. Various genera of salt-tolerant plant growth promoting *Rhizobacteria*(ST-PGPR) have been isolated from extreme alkaline, saline, and sodic soils. Many of them are also known to mitigate various biotic and abiotic stresses in plants. The genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Agrobacterium*, *Streptomyces*, *Klebsiella*, and *Ochromobacter* are best reported for improving the productivity of diverse crops under saline conditions (Oliveira *et al*., 2015) [12].plant hormones like GA3, ABA and IAA showed that these hormones ameliorated high concentration of salt stress when halotolerant strains like *P. chlororaphis* and *P. Extremorientalis* was inoculated in the soil which ultimately restored and improved growth (Egamberdieva, 2011) [8]. Halophilic and halotolerant bacteria can be used for the production of enzymes with different immunological properties (Shirazian *et al*., 2016) [16] and also essential for nutrient recycling and for maintaining the soil health in a salty environment.

Materials and method
Sample collection
Soil sample was collected from the depth of 10-12 inch around Perambalur in a sterile polythene bag and samples were kept at room temperature until used. Soil analysis was conducted to measure electro conductivity and pH.

Isolation and Screening of Bacteria
Soil suspension was prepared with 1g of soil in 10ml of sterile double distilled water and vortexes then diluted upto 107.
Cultivable bacterial strains were isolated and enumerated. Isolation of bacteria was carried out with Tryptic soya agar (TSA) with 5% sodium chloride and YEMA agar adopting pourplate technique and incubated overnight at 37 ± 2 °C for 2–3 days.

Optimal salinity checking (Caton *et al*., 2004) [5].
Each colony of the plates were added to 500 ml of liquid nutrient medium with 5,10,15,20, and 25% NaCl and kept overnight under shaking incubation then incubated under static condition at 37 ± 2 °C for 48 h. Broth cultures were subject to Optimal growth by measuring OD at 600 nm.

Chromium reduction test
About 25 mL of the broth was centrifuged at 4000 rpm for 15 min and the supernatant was used for the estimation of chromium. To 1 mL of the supernatant, 9 mL of 0.2 M sulphuric acid and 0.2 mL of 0.25% diphenylcarbazide in acetone were added, and the absorbance of the pink colour developed was read at 540 nm using distilled water as blank. The linear regression of the standard graph was used for the estimation of chromium present in the solution. The chromium removal percentage was calculated using the following formula
E = (Ci - Cf / Ci) × 100 Where, E = Percentage removal of heavy metal; Ci = initial metal ion concentration, mg/L; Cf = final metal ion concentration, mg/L

Biochemical characterization
Biochemical analysis of isolates were carried out by IMViC, catalase,oxidase and genera were identified according to Bergey's Manual of Determinative Bacteriology and classified primarily through morphological, physiological and biochemical observation.

Assessment of PGP Traits
Indole-3-acetic acid (IAA) Production
The selected antagonistic bacterial strains were grown in 25ml of nutrient broth medium f or 24 hrs. After incubation period, bacterial culture were harvested and centrifuged at 10000xg for 15min at 4°c. Two drops of orthophosphoric acid were added to 2ml of cell free supernatant and the development of colour was observed the presence of a pink colour indicate positive reaction for indole acetic acid and yellow colour indicate negative reaction.

Phosphate solubilization
The isolates was checked using Pikovskaya's agar medium (Hi Media, Mumbai). In these medium added 0.5gm tricalcium phosphate, 0.12gm yeast extract, 2.5g dextrose, 0.125gm ammonium sulphate, 0.05gm potassium chloride, 0.025gm magnesium sulphate, 0.000025gm manganese sulphate, 0.000025g ferrous sulphate and 3.75g agar powder.
Then sterilized the medium in autoclave. Then poured the medium in all the sterile petri plates. Next day put the 2µl of drop in all the plates. Incubated all the plates in incubator at 28-30oC for 6- 7 days. Then observed the zone of phosphate solubilization.

Siderophore test
3 mg CAS was dissolved in 50 ml glass distilled water and mixed with 10 ml of iron (III) solution (1mM FeCl3.6H20 in 10mM HCl). This was added to 72.9 mg of hexadecyltrimethyl ammoniumbromide (HDTMA) in 40 ml of distilled water. The CAS reagent was then autoclaved for 15 minutes. This reagent was added to nutrient agar medium. The solidified CAS agar plates were inoculated with test bacterial strain.

Ammonia production test
To research the production of ammonia, each identified *Rhizobacteria* strain was grown in peptone broth (10 mL) and incubated at °C for 48 to 72 h. After incubation, 0.5 mL of Nessler's reagent was added to bacterial suspension. The development of brown to yellow color indicated ammonia production.

Salt aggregation test
For the determination of microbial cell surface hydrophobicity (CSH), the salt aggregation test (SAT) was used. Bacterial cells from agar-grown cultures were washed twice with 0.01 M sodium phosphate buffer (pH 7.2) containing approximately 109 bacterial cells ml−1 (with an absorbance at 540 nm of approximately 1.0). Equal volumes (1000 µl) of ammonium sulfate diluted in different M, sodium phosphate buffer (pH 6.8) and bacterial suspensions were mixed in wells. Incubation time for tubes at room temperature was extended to 3 h. SAT was defined as positive (+) if bacterial aggregation was clearly visible and negative (−) if no aggregation was observed; the concentrations of ammonium sulfate at which aggregation appeared was then registered. The SAT titre is defined as the lowest concentration of ammonium sulfate at which bacteria still show clearly visible cell aggregation. The strains were tested for auto-aggregation in phosphate buffer.

Testing of bacterial culture and salinity stress on plant
Inoculum preparation
Bacterial culture was prepared by inoculating pure single colony of nutrient broth and incubated at 30 ± 1° C for overnight (12 h). The bacterial cells were harvested by centrifugation at 5000rpm for 5 min. The pellet obtained was re-suspended in sterilized distilled water. The optical density of the bacterial culture was maintained at 107 CFU mL- 1OD at 600 nm.

Seed treatment
10^9 bacterial cells ml^{-1} (with an absorbance at 540 nm of approximately 1.0) bacterial suspension was mixed with known volume of rice water at 1:1 ratio and used to inoculate vigna radiate seeds. Seeds were soaked on culture suspension and allowed to air dry. Treated Seeds were then kept under 5% salt condition to check the rate of germination. Seeds treated under water used as control.

Plant material and growth conditions
For the pot experiment, soil was collected from the botanical garden. The soil was sieved (2 mm) and sterilized by autoclaving at 121° C for 15 min at 15 psi. Pots were treated with *Bacillus sp* and *Rhizobium* sp alone and flooded with salt water (5%). Then the pots were planted with 5cm tall paddy plant. All the pots were kept under natural light (sunlight) with average 37/27° C day and night temperature and humidity 30 % during the study period. The pot experiment without culture served as control. After 20 days, trunk length, root length (cm), weight of dried and original sample.

Plant growth attributes (Cesaro *et al*., 2015) [6]
Initially seed germination was recorded. At maturity, three replicates from each treatment were harvested to measure the length of root and shoot after 10 days. The following

contributing parameters were also taken from the remaining plants of each treatment

SG= Number of germinated seeds/Number of total seeds X 100%

Vigor index = RL+SL×GP

where RL is root length (cm), SL is shoot length (cm) and GP is germination percentage.

Results and discussion

Isolation and testing of salt tolerant bacteria

The collected agro ecological zone soil type investigated in this study present a varied microbial density. The rhizosphere of soil zone contains mesophilic microflora 15 × 10^7 CFU/g on TSA agar and 12x10^7 in YEMA plates. Conventional methods based on biochemical and phenotypic techniques for the identification. Isolated PF1 found to be Gram-positive slender rods spore bearing *Bacilli* showed positive on indole, MR, catalse and oxidase. Salt tolerant bacteria from paddy field soil was isolated by crowded plate method and identified as Bacillus sp capable to tolerate 20% salinity. Isolate PF 2 Viscous colonies on YEMA plates are Gram negative rod positive on indole, catalase and oxidase. Based on biochemical character (Table 1) selected bacteria genera are identified as *Bacillus* sp, *and Rhizobium* sp. The ability of bacteria grow in various salt concentration is given in (Fig 1). Among the isolates, *Bacillus s*howed significant growth in all tested salt concentration. Maximum growth obtained at 5% of Nacl the growth rate is 1.24 OD. There was moderate growth at 20% of Nacl the growth rate is 0.84 OD. Growth of *Bacillus* sp at 25% salt showed less significant growth. Tested *Rhizobium* showed significant growth at only 5 % of salt concentration recorded growth rate is 1.18. No growth was observed at 10%. Further isolate found to reduce chromium VI to III and reduced 72% of chromium reduction. Aleem *et al* (2003) [2] reported that the soil pollution with heavy metals could lead to the appearance of heavy-metal resistant PGPR in the soil.The diversity of salt-tolerant bacteria isolated from paddy rhizosphere in Taoyuan, China was reported by Zhang *et al*., (2018) [19]. They found that most of the isolates were able to tolerate up to 8% NaCl and belong to the genus *Bacillus*. Kantha et al. (2015) [11] reported Potential of biofertilizers from selected *Rhodopseudomonas palustris* strains to assist rice (Oryza sativa L. subsp. indica) growth under salt stress. Ahmad *et al*., (2011) [1] evaluated the combined application of salt-tolerant Rhizobium and Pseudomonas under salt-stressed conditions for improving the productivity of mungbean. Plant growth promoting traits of isolates on IAA production, Phopshate solubilization test, Siderophore production, and Ammonia production were shown in (Table 2). Out of 2 tested, siderophore production was noted only in *Bacillus sp*. Phosphate soluilization and IAA production test was positive on *Bacillus* and *Rhizobium*, The ammonia was noted only on *Bacillus* (Table 2). Zahir *et al*., (2010) [10, 18]. Isolated *Rhizobium phaseoli* strains from the mung bean nodules, and, the most salt tolerant and high auxin producing rhizobial isolate improving growth and yield of mung bean under saline conditions in a pot experiment. Isolated and stated that *Bacillus* sp. increased growth and development of maize under drought and salinity through accumulation of proline and soluble sugars (Alori *et al*., 2017) [3].

Research has shown that IAA aids in the plant adaptation to salinity stress, and enhances the root and shoot growth under salinity and heavy metal stress (Fahad *et al*., 2015) [9]. All the isolates were subjected to salt aggregation test (Table 2) the ability of Bacteria aggregate the ammonium sulphate in various concentration in mol. The *Rhizobium* is well aggregate the salt at 0.1 M and moderate at 0.3M. The *Bacillus* is well aggregate the salt at 0.2 and Moderate at 0.4.

The lowest concentration of ammonium sulphate giving a visible aggregation for *Bacillus* sp was 0.2 M and 0.1 M for *Rhizobium* sp scored as the SAT hydrophobicity value. The impact of green gram seed treated with isolated bacteria culture of *Bacillus*, and *Rhizobium* bacterial culture under the 3% of Nacl concentration is given in table 3. After 10 days the *Bacillus sp* and *Rhizobium* treated seed is well grown in the salt concentration with relative seed germination 40 and 30% with VI of 508 and 309 respectively monoculture treatment. No growth appearance in the control. The *mixed culture* treated seeds shows good seed germination was estimated as 70 % and the vigor index is 1106. Salinity is considered a limiting factor in nodulation and leghaemoglobin content in legume- Rhizobium associations, which can adversely affect the yield of plants (Vishal *et al*. 2013) [17] reported that the inoculation with *Rhizobium* culture had invariably and significantly promoted plant growth under salinity soil.

Table 1: Biochemical characters of isolated colonies

Biochemical characters	PFI1 *Bacillus sp*	PFI2 *Rhizoium* sp
Colony morphology	Rhizoidal colony	Mucoid colony
Grams stain	Positive rod	Negative rod
Indole	+	+
MR	+	-
VP	-	-
citrate	-	-
KOH	-	+
Catalase	+	+
Oxidase	+	+
Cr absorption	-	-

Table 2: plant growth parameters and Salt aggregation test (SAT) on isolated colonies

S.NO	Tests	*Bacillus* sp	*Rhizobium* sp
1.	IAA	Positive	Positive
2.	Siderohore	Positive	Negative
3.	Phosphate test	Positive	Positive
4.	Ammonia	Positive	Negative
5.	SAT 0.1 M	++	++
6.	SAT 0.2 M	++	+
7.	SAT 0.3 M	+	+
8.	SAT 0.4 M	+	_
9.	SAT 0.5 M	_	_
10.	1 M	_	_

Table 3: Seed germination test under 3% salinity (out of 10seeds)

S.NO	Treatment group	Number Of Seed germinated	GI (%)	Shoot + root length	VI
1	Control	--	--	--	--
2	*Bacillus* sp	4	40	12.7	508
3	*Rhizobium* sp	3	30	10.3	309
4	Mixed	7	70	15.8	1106

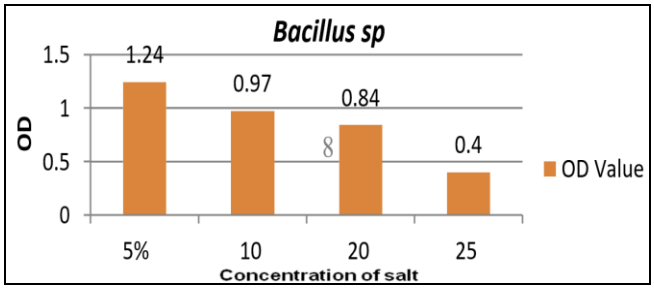

Fig 1: Growth rate of Bacillus in various salt concentration

Conclusion

Co inoculation of Bacillus sp and Rhizobium sp protect and stumilate plant growth under high salt condition

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