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Plant growth promotion and alleviation of salts in rice by *Paenibacillus* spp

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Abstract
Soil salinity plays an evil role by making a crop highly fragile and thereby deteriorating its productivity. Rice is a salt sensitive crop and soil salinity is the single most widespread soil toxicity problem facing rice production. Major problem soils under which rice is grown are Sodic soils, Saline soils (includes inland and coastal saline soils) and Saline-sodic soils and these soils adversely affect growth and productivity of rice. In this study, *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 were evaluated for alleviating salt stress. The isolates could tolerate salinity stress up to 15% NaCl. The isolates possessed traits like nitrogen fixation, phosphorous solubilisation, siderophore and IAA production. The two isolates were taken for gnotobiotic assay under salt stressed conditions. A considerable growth of rice seedling (CO-51) was noted under gnotobiotic assay. The pot culture studies were performed under different salt stress (0 mM NaCl, 50 mM NaCl, 100 mM NaCl). Among various treatments, the treatment T5 (*Paenibacillus castaneae* VPB1 + 50 mM NaCl) recorded maximum shoot length of 19.7 and 56.0 cm and root length of 18.6 and 37.5 cm at 30 and 60 DAS respectively. *Paenibacillus castaneae* VPB1 plus 50 mM NaCl significantly increased the number of grains (201 plant^{-1}) treatment without bacterial inoculation with salt stress. Hence, it is concluded that *Paenibacillus* spp. could be recommended as potential bio-inoculant to rice crop under salt stressed conditions after field evaluation.

Keywords: *Paenibacillus*, rice, salt stress, gnotobiotic conditions, PGPR

Introduction
Increased concentration of chlorides and sulphates of calcium, magnesium and sodium salt content in soil is termed to be soil salinity. It plays an evil role by making a crop highly fragile and thereby deteriorating its productivity. High osmotic pressure of soil solution containing excess soluble salts lowers the available water content to the plants thereby affecting either its macromolecular structure or physiology. As a result of salinity, soil integrations, farming profitability and its monetary returns are further affected. (Hu and Schmidhalter, 2002).
Rice grown as a staple cereal crop in India, is largely affected by the prevalence of these white alkali soils. It highly retards the crop growth rate during transplanting, blooming and harvest stages. It produces sterile tillers, prolific ripened panicles and lowers the amount of filled grains, 1000 grain weight during regenerative phase (Asch *et al*., 2000) [1].
It is evident from a great number of reports that certain bacterial strains are beneficial for the growth of plants. A group of bacteria that display such effects are called plant growth-promoting rhizobacteria (PGPR). Accumulation of these compatible solute molecules serves as a salt tolerant indicator in plants and bacteria (Gul & Khan, 2008) [3]. Osmolytic accumulation in cells under salt stress supported the growth and survival of both bacteria and plants. Bacterial root colonization and crop growth were further enhanced due to secondary metabolite accumulation in plants under saline stress (Karlidag *et al*., 2011). At present to mitigate the terminal water stress of rice plants, one phyllosphere bacterium Pink Pigmented Facultative Methylotrophs (PPFM) is recommended. However, this PPFM works after establishment of crop in the field. There is no suitable bio inoculant to alleviate the salt stress imposed onset of seed germination. In this research, halotolerant bacterial isolates *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 were employed for evaluating rice crop under salt stress.

Materials and methods
Growth of halo tolerant bacterial isolates at different NaCl concentration
The two halo tolerant bacterial isolates *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 were analyzed for their growth in trypticase soy brothsupplemented with different concentration of NaCl ranging from 0%, 5%, 10%, 15%, 20% and 25% NaCl.

The population of *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 isolates were detected by serial dilution technique in trypticase soy agar (TSA) plates containing 15% NaCl.

ARA activity

Nitrogen fixing efficiency of halo tolerant bacterial cultures were evaluated through acetylene reduction assay (ARA) as described by (Turner *et al.*, 1980) [16]. Twenty ml of Trypticase Soya broth was prepared in Gas chromatography bottles supplemented with 2.571 M NaCl and without NaCl for maintaining stress and controlled conditions, respectively. The air in bottles was evacuated and 5ml of 10% pure acetylene gas was injected. The bottles were incubated for 24 h at room temperature. After incubation, 2 ml of gas sample was withdrawn and injected into the Gas chromatograph fitted with porapack Q column and FID detector. The acetylene reduction activity was expressed as n moles of ethylene produced h^{-1} mg^{-1}cell protein.

Phosphate solubilization

Phosphate solubilizing ability of each halo tolerant bacterial isolate was observed on Pikovskaya (PVK) (Pikovskaya, 1948) [11] medium plates with and without 2.57 M NaCl concentration for maintaining stress and controlled conditions, respectively. PVK containing five gram of Tri-calcium phosphate (TCP) as sole phosphorus source (Mehta and Nautiyal, 2001) [7]. The growth of bacterial isolates was analysed by spotting10 µl of cultures on medium, incubated at 30°C for 7 days. The ability of the bacteria to solubilize insoluble phosphorus and form clear halo zones around them was considered as positive result for phosphate solubilization potential. The solubilization index was calculated using the formula (Sarkar *et al.*, 2018) [14].

Production of indole acetic acid (IAA)

The production of IAA by the endophytic bacterial isolates was determined according to the method of (Patten and Glick, 2002) [10]. Bacterial isolates were inoculated into 5 ml LB broth containing 0.2% L-tryptophan, pH 7.0 and incubated at 28˚C with shaking at125 rpm for 7 days. The cultures were centrifuged at 11,000 rpm for 15 min. One ml of the supernatant was mixed with 2 ml of Salkowski's reagent and the appearance of a pink colour indicated IAA production. Optical density (OD) was read at 530 nm using micro plate reader. The level of IAA produced was estimated against a standard IAA.

Siderophore production

The siderophore production test in the bacterial isolates was done using the Chromazural S (CAS) agar plates. CAS indicator solution (60.5 mg of chrome azurol S was dissolved in 50 ml of double-distilled (dd) water to which 10 ml of Fe III solution and 40 ml of HDTMA solution) of was prepared and sterilized. Basal Agar medium (3 g of 0.1 M 3-(morpholino) propane sulphonic acid (MOPS), 0.05 g L-Aparagine, 0.05 g of NaCl, 0.01 g of ammonium chloride (NH3Cl) and 0.03 g of KH_2PO_4, 2g Agar were added and dissolved in distilled water) was prepared with and without salt. The medium was then autoclaved for 15 min at 121°C and 15 lb pressure. The glucose solution (50%) was prepared and autoclaved. Once cooled, 2ml of the 50 per cent glucose solution was added to the autoclaved basal agar medium. Then, 10 ml of the CAS indicator solution was added carefully along the walls of the flask with constant stirring. Once mixed thoroughly, the resulting CAS agar medium (100 ml) was plated in sterile plates. 10 µl of 24h old fresh culture was spotted on CAS agar plates with and without NaCl and incubated for 48h at 30° C. The production of siderophore was indicated by the appearance of orange halo around the spotted colonies. The siderophore production was calculated by the following formulae,

$$\text{Siderophore\%} = \frac{\text{Halozone diameter}}{\text{Colony diameter}}$$

Germination test and vigour index

Paddy seeds were surface sterilized with 70% ethanol and imbibed in 24 h old halo tolerant bacterial suspension for 4 h. Then the seeds were placed in germination paper (Mia and Shamsuddin, 2009) supplemented with different NaCl solutions (0 mM, 50 mM and 100mM) and incubated for 14 days at 30±2 ˚C to assess the germination percentage and the vigour index. Vigour index was calculated on 15 DAS using the following formula.

Vigour index = Germination percent × Plant height.

Pot culture study

For pot culture experiment black clay loam textured soil was collected from wet land of Agricultural College and Research Institute, Coimbatore - 641003. Soil pH (8.72), EC (0.47 dS m-1), N (166.52 kg ha-1), P(27.60 kg ha-1) and K (310.7 kg ha-1) was determined. Soil was thoroughly mixed to break any clods present and filled in plastic pots of 15 cm diameter at the rate of 4 kg per pot. Plastic pots were chosen deliberately to minimize leaching loss. The plastic pots holes were sealed with cotton and wax in order to prevent the water loss frompot holes. Electrical conductivity (EC) of soil was calibrated to reach 50 mM and 100 mM. Control pots were kept to check, if there was a decline in level of soil electrical conductivity which directly corresponds to salt concentration level. Surface sterilization and seed imbibition were performed as described in previous section.

Statistical analysis

The data obtained from different experiments were statistically analysed using AGRES software through ANOVA for completely randomized block design.

Results and discussion

Growth of halotolerant bacterial isolates at different NaCl concentration

The two halo tolerant bacterial isolates *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 were amended with different concentration of NaCl (0%, 5%, 10%, 15%, 20% and 25%) in Trypticase Soy agar and analysed for their growth pattern and bacterial count. The results showed that the population of both *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 upto 15% NaCl. Population count of *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 was analysed in 15% NaCl. Results showed the lesser population count only at 9 and 7 $CFUml^{-1}$ respectively were noted. Similarly, *Bacillus* and *Hallobacillus* strains were screened using different NaCl concentration (Ramdoss *et al*., 2013) [12]. Roohi *et al.* (2012) [13] isolated halotrophic bacteria from Karak salt mines of Pakistan. These authors used salt concentrations of 5-20% of NaCl.

Estimation of ARA activity

ARA activity was analysed for the two selected bacterial strains *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5. Among these two isolates tested, *Paenibacillu scastaneae* VPB1 showed higher activity at 2.57 M NaCl concentration of about 2.06 nM h^{-1} mg^{-1} cell protein and without NaCl (1.31 nM h^{-1} mg^{-1} cell protein) followed by

Paenibacillus stellifer KVPB 51.76 nM h-1 mg-1 cell protein. (Table 1).

Assay for phosphate solubilization

The phosphate solubilizing ability of halo tolerant bacterial isolates was tested in Pikovaskya's medium supplemented with 0.1% tricalcium phosphate (TCP). The results showed that, in the absence of NaCl, maximum halozone was exhibited by *Paenibacillus castaneae* VPB1 (2.32) followed by *Paenibacillus stellifer* KVPB5 (2.05) whereas at 2.57 M NaCl, the phosphate solubilizing ability of *Paenibacillus castaneae* VPB1 recorded 2.05 and for *Paenibacillu stellifer* KVPB5 2.06. Similarly, Sharma *et al.* (2016) [15] reported halotolerant rhizobacteria *Klebsiella* sp. which solubilized 55.6 µg phosphate mg^{-1} dry weight in Pikovaskyas broth containing 0.1% TCP (Table 1).

Assay for indole acetic acid (IAA) production

The ability of halo tolerant bacterial isolates to produce indole acetic acid was observed in Tryptic Soy broth amended with tryptophan as precursor. The results showed that amendment with tryptophan, the halo tolerant bacterial isolate *Paenibacillus castaneae* VPB1 exhibited maximum production of 29.36 µg ml^{-1} followed by *Paenibacillus stellifer* KVPB5 of about 26.68 µg ml^{-1} without the amendment of NaCl. Whereas, when supplemented with 2.57 M of NaCl, the isolate *Paenibacillus castaneae*VPB1 showed maximum production of 11.21 µg ml^{-1} followed by *Paenibacillu stellifer* KVPB5 which had IAA production of 10.20 µg ml^{-1}. (Table 1).

Siderophore production assay

The halo tolerant bacterial isolates were tested siderophore production using change in colour of chromo azurol S (CAS) reagent. The results depicted that maximum percent of siderophore was noticed by *Paenibacillus castaneae* VPB1 (50.28) followed by *Paenibacillus stellifer* KVPB5 (45.84). Whereas, in the presence of 2.57 M NaCl, *Paenibacillus castaneae* VPB1 exhibited maximum percent of siderophore (42.09) followed by *Paenibacillu stellifer* KVPB5 (32.84). Masum *et al.* (2018) showed halotolerant bacteria *Bacillus velezenis* NRRL B-41580 and *Bacillus siamensis* KCTC 13613 exhibited the siderohpore production (Table 1).

Table 1: Plant growth promoting activities of *Paenibacillus* spp. under salt stressed conditions

S. No.	Halotolerant isolates	AARA (Ethylene produced nM h^{-1} mg^{-1} cell protein		Indole acetic acid production with L-tryptophan (µg ml^{-1})		Siderophore Production in (%) units	
		Without NaCl	With 2.57 M NaCl	Without NaCl	With 2.57 M NaCl	Without NaCl	With 2.57 M NaCl
1.	*Paenibacillus castaneae* VPB1	1.31 ± 0.02	2.06 ± 0.04	29.36 ± 0.50	10.35 ± 0.13	50.28 ± 0.22	42.09 ± 0.28
2.	*Paenibacillus stellifer* KVPB5	0.67 ± 0.06	1.76 ± 0.00	26.68 ± 0.72	8.74 ± 0.14	45.84 ± 0.59	32.84 ± 0.20

Values are mean (± standard error) (n=3)

Germination test and vigour index

The two halo tolerant bacterial isolates *viz*., *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 along with uninoculated control were evaluated for tolerance against salinity stress in paddy variety CO51. The data recorded on germination, vigour on 14th day of crop growth after imposing 0, 50 mM and 100 mM of NaCl stress were presented in Table 2. The results showed that the germination percentage was found to maximum in treatment *Paenibacillus castaneae* VPB1 imposed with 50 mM NaCL of 90.29% followed by *Paenibacillus stellifer* KVPB5 imposed with 50 mM NaCL of 85.78%. Similarly, the vigour index was found to be maximum in treatment with *Paenibacillus castaneae* VPB1 imposed with 0 mM NaCl of 2108 followed by *Paenibacillus castaneae* VPB1 imposed with 50 mM NaCL of 1821, respectively.

Table 2: Germination and vigour index of rice seedlings under salts stressed conditions inoculated with *Paenibacillus* spp.

S. No.	Treatments	Germination%	Vigour index
T1	Control + 0 mM NaCl	100	1892
T2	Control + 50 mM NaCl	88.30	1689
T3	Control + 100 mM NaCl	50.25	1582
T4	*Paenibacillus castaneae* VPB1 + 0 mM NaCl	100	2108
T5	*Paenibacillus castaneae* VPB1 + 50 mM NaCl	90.29	1821
T6	*Paenibacillus castaneae* VPB1 + 100 mM NaCl;	65.75	1728
T7	*Paenibacillus stellifer* KVPB5 + 0 mM NaCl;	100	1792
T8	*Paenibacillus stellifer* KVPB5 + 50 mM NaCl;	85.78	1628
T9	*Paenibacillus stellifer* KVPB5 + 100 mM NaCl	67.28	1527

Pot culture study

The two best performing halo tolerant bacterial isolates *viz*., *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 along with uninoculated control were evaluated for tolerance against salinity stress in paddy variety CO51 using pot culture experiment and the plant biometric observations were recorded.

Shoot length was observed on 30th and 60th days interval. The inoculated treatments showed increased shoot length at both 30 and 60 DAS than the un-inoculated treatments. Among various treatments, the treatment T5 (*Paenibacillus castaneae* VPB1 + 50 mM NaCl) recorded maximum shoot length of 19.7 and 56.0 cm at 30 and 60 DAS, respectively and the results were depicted in Table 3.

Among the plant biometric observations, the root length parameter was observed at 30th and 60th DAS. The result showed that the treatment T5 (*Paenibacillus castaneae* VPB1 + 50 mM NaCl) showed increased root length of 18.6 and 37.5 cm at 30 and 60 DAS respectively, compared to un-inoculated treatments (Table 4).

Plants samples from all the treatments were collected dried in oven and recorded separately. Dry weight of the root and

shoot are presented in (Table 5). The results depicted that compared to control, the inoculated treatment T8 (*Paenibacillus stellifer* KVPB5 + 50 mM NaCl) showed maximum root and shoot dry weight of 3.89 and 5.12 g^{-1} plant respectively. The data recorded on total number of grains per plant at 90 DAT are furnished in Table 6. The result depicted that *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 treated plants were reported to have more number of grains per panicle. Nautiyal *et al*. (2013) [9] reported that *Bacillus amyloliquefaciens* SN13 increased the root and shoot length at 200 mM salt stress in rice. Choi *et al.* (2016) [2] reported that *Paenibacillus yongiensis* DCY84^T increased germination and vigour of the rice cultivars.

Table 3: Effect of inoculation of *Paenibacillus* spp. on rice shoot length under salt stressed conditions

S. No.	Treatments	Shoot length (cm) at different DAT	
		30 days	60 days
T1	Control + 0 mM NaCl	20.0±0.25^b	56.2±0.75^b
T2	Control + 50 mM NaCl	18.3±0.15^e	54.6±0.92^e
T3	Control + 100 mM NaCl	16.9±0.45^h	52.1±0.62^i
T4	*Paenibacillus castaneae* VPB1 + 0 mM NaCl	21.2±0.26^a	57.4±0.58^a
T5	*Paenibacillus castaneae* VPB1 + 50 mM NaCl	19.7±0.19^c	56.0±0.46^c
T6	*Paenibacillus castaneae* VPB1 + 100 mM NaCl;	18.1±0.32^g	54.0±0.24^b
T7	*Paenibacillus stellifer* KVPB5 + 0 mM NaCl;	21.0±0.24^a	58.±0.84^d
T8	*Paenibacillus stellifer* KVPB5 + 50 mM NaCl;	19.1±0.13^d	54.2±0.72^h
T9	*Paenibacillus stellifer* KVPB5 + 100 mM NaCl	18.5±0.28^f	53.8±0.56^g

Data represent the mean ± SE from three replicates. Different letters in the same column indicate significant differences according to Duncan's test ($P < 0.05$).

Table 4: Effect of inoculation of *Paenibacillus* spp. on rice root length under salt stressed conditions

S. No.	Treatments	Root length (cm) at different DAT	
		30 days	60 days
T1	Control + 0 mM NaCl	19.5±0.23^c	38.5±0.46^c
T2	Control + 50 mM NaCl	17.2±0.56^f	35.2±0.58^f
T3	Control + 100 mM NaCl	15.3±0.35^h	31.6±0.64^i
T4	*Paenibacillus castaneae* VPB1 + 0 mM NaCl	20.4±0.42^b	39.3±0.52^a
T5	*Paenibacillus castaneae* VPB1 + 50 mM NaCl	18.6±0.35^d	37.5±0.35^d
T6	*Paenibacillus castaneae* VPB1 + 100 mM NaCl;	16.3±0.62^g	33.4±0.61^h
T7	*Paenibacillus stellifer* KVPB5 + 0 mM NaCl;	21.0±0.24^a	40.1±0.49^b
T8	*Paenibacillus stellifer* KVPB5 + 50 mM NaCl;	18.1±0.41^e	36.2±0.25^e
T9	*Paenibacillus stellifer* KVPB5 + 100 mM NaCl	16.7±0.37^g	32.0±0.46^g

Data represent the mean ± SE from three replicates. Different letters in the same column indicate significant differences according to Duncan's test ($P < 0.05$).

Table 5: Effect of inoculation of *Paenibacillus* spp. on rice shoot and root dry weight under salt stressed conditions

S. No	Treatments	Root dry weight (g/plant)	Shoot dry weight (g/plant)
T1	Control + 0 mM NaCl	4.22±0.22^b	5.62±0.32^c
T2	Control + 50 mM NaCl	3.81±0.34^f	4.98±0.15^f
T3	Control + 100 mM NaCl	3.40±0.41^i	4.12±0.23^i
T4	*Paenibacillus castaneae* VPB1 + 0 mM NaCl	4.35±0.36^a	5.71±0.24^b
T5	*Paenibacillus castaneae* VPB1 + 50 mM NaCl	3.91±0.53^d	5.02±0.12^d
T6	*Paenibacillus castaneae* VPB1 + 100 mM NaCl;	3.56±0.21^h	4.32±0.32^g
T7	*Paenibacillus stellifer* KVPB5 + 0 mM NaCl;	4.39±0.33^c	5.82±0.15^a
T8	*Paenibacillus stellifer* KVPB5 + 50 mM NaCl;	3.89±0.42^e	5.12±0.42^e
T9	*Paenibacillus stellifer* KVPB5 + 100 mM NaCl	3.52±0.13^g	4.29±0.26^h

Data represent the mean ± SE from three replicates. Different letters in the same column indicate significant differences according to Duncan's test ($P < 0.05$).

Table 6: Effect of inoculation of *Paenibacillus* spp. on grain yield of rice under salt stressed conditions

S. No.	Treatments	Total no of grains per plant
T1	Control + 0 mM NaCl	215±3.2^b
T2	Control + 50 mM NaCl	192±4.2^f
T3	Control + 100 mM NaCl	179±2.5^i
T4	*Paenibacillus castaneae* VPB1 + 0 mM NaCl	220±3.5^a
T5	*Paenibacillus castaneae* VPB1 + 50 mM NaCl	201±4.6^d
T6	*Paenibacillus castaneae* VPB1 + 100 mM NaCl;	183±2.8^h
T7	*Paenibacillus stellifer* KVPB5 + 0 mM NaCl;	208±3.2^c
T8	*Paenibacillus stellifer* KVPB5 + 50 mM NaCl;	196±1.8^e
T9	*Paenibacillus stellifer* KVPB5 + 100 mM NaCl	187±2.6^g

Data represent the mean ± SE from three replicates. Different letters in the same column indicate significant differences according to Duncan's test ($P < 0.05$).

Two efficient *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 were isolated from rice rhizosphere soils grew in 15% salt stress and were able to fix nitrogen and solubilize phosphorus. A reasonable seedling growth were observed in gnotobiotic and pot culture study on inoculation with *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5. Salt tolerant mechanisms by these two isolates may be studied in future. Also, accumulation of osmolytes, electrolyte leakage of plants with and without bacterial inoculation may be studied under field conditions. Field evaluation may be conducted for the two efficient *Paenibacillus castaneae* VPB1 and *Paenibacillus stellifer* KVPB5 isolates before recommended to farmers.

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