Chapter 2

Effect of salt stress on PGPR traits of PGPR strains individually and in consortia

2.1. Introduction

Multiple mechanisms, such as phosphate solubilization, di-nitrogen fixation, antifungal activity, phytohormone production and siderophore biosynthesis etc. are responsible for the plant growth promotion and increased yield (Bashan & Holguin, 1997). Currently there is an increasing trend to use mixed populations of microbial bioinoculants so that many beneficial traits can have combined effect on plant growth as compared to individual strains (Santoyo et al., 2021). For an efficient consortium, it is necessary for the strains to have varied abilities for PGPR effect and survival in different stressed conditions along with synergistic effect over individual strains. Many studies have been conducted showing the beneficial effect of applying PGPR consortia for the alleviation of salinity stress (Shilev, 2020).

Many PGPR traits are known to be beneficial in promoting plant growth under salt stress. Indole-acetic acid (IAA) produced by the PGPR has been reported to mitigate salt stress and stimulate plant growth (Meena et al., 2020). (Li et al., 2020) have reported salt stress amelioration in plants inoculated with a halotolerant strain by regulating the phytohormone levels. Plants inoculated with IAA producing PGPR consortia demonstrated higher plant growth promotion in comparison to individual strains (Kumar et al., 2017; Kumawat et al., 2021). Sultana et al., (2021) have reported salt tolerant siderophore producing strains supporting plant growth in saline soils with iron limitation. Biofilm formation is another important trait which protects plants against the osmotic stress by binding Na^+ ions promotes stronger adherence to root surface and mixed biofilms can result in enhanced bacterial interaction within biofilm and promote consortial PGPR activity in stressed conditions (Gupta et al., 2017). Studies have shown several *Pseudomonas* sp. with salt tolerance displaying several antagonistic abilities such as HCN, siderophore production along with salt stress alleviation and biocontrol activity in plants (Cabanás et al., 2018; Kotasthane et al., 2017; Pandey & Gupta, 2020).

Synergistic effects of co-inoculating rhizospheric bacteria which enhanced the phosphate content in plants have been reported (Emami et al., 2020; P. Kumar et al., 2016; Zineb et al., 2019). Kumar et al. (2016) have reported growth and yield improvement in plants inoculated with siderophore producing PGPR in consortia with *Rhizobium* strains. Non-rhizobial PGPR such as *Pseudomonas* sp., *Enterobacter* sp. in consortium with rhizobia have been subject of several studies for consortial synergy and plant growth promotion (Jha & Saraf, 2012; Kumawat et al., 2021, 2019; Menéndez & Paço, 2020), abiotic stress alleviation (Belimov et al., 2020; Shilev, 2020), biocontrol activity (Kumar et al., 2016).

As discussed by Sanchez-Gorostiaga et al., (2019), community functions depend on separate contribution from each species and their interactions, in the absence of interaction this function could be simply additive while communicative interactions can also be dominated by pair-wise or even higher interactions as well. Thus, before establishing the effectiveness of a consortium for plant growth promotion it is necessary to understand the microbial interactions amongst the PGPR themselves as well as under the stress condition in question.

This chapter deals with the investigation of the ability of bacterial strains to demonstrate PGPR traits under salt stress and the effect of consortia development for enhancing the stress tolerance for producing these traits. Three classes of PGPR strains were used and one strain from each group of PGPR was chosen to prepare 18 different consortia which were then analysed for the various PGPR traits in different salt concentrations and to establish synergy among the consortial members under stressed conditions.

2.2. Materials and Methods

2.2.1. Maintenance of microbial cultures and growth conditions:

Microbial strains under study were as listed in Table 2.1. The bacteria were routinely maintained as follows - *Enterobacter* sp. C1D on Luria Bertani (LB) medium (Hi-Media, India), *Pseudomonas* spp. on pseudomonas agar medium and the *Rhizobium* sp. on YEMA medium (Appendix I). NGR234 was maintained on Tryptone yeast (TY) medium (Vora et al., 2021) amended with rifampicin (50µg/ml). Bacterial growth was conducted at 30˚C throughout the study. The glycerol stocks were maintained at -20˚C and bacterial cultures were revived from the glycerol stocks at every 6 months intervals. Fungal strains *Fusarium udum* and *Rhizoctonia solani* were maintained on potato dextrose agar (PDA, Hi-Media, India) and stored at 4˚C

Table 2.1: Microbial strains used in the present study

PGPR Strains (Shortened names used)	**Source (Isolation)**	**PGPR Traits**	**Reference**
Enterobacter sp. C1D (EC1D)	Industrial waste effluent near Sarod, Mahi river estuarine, Gujarat, India	Heavy metal tolerant, phosphate solubilization IAA production, ACCd production	Subrahmanyam et al., 2018
Pseudomonas protegens CHA0 (formerly called *P. fluorescence* CHA0) (PCHA0)	Tobacco rhizosphere	Production of bioactive metabolites such as DAPG, pyoluteorin, pyrrolnitrin, Siderophores pyochelin and pseudobactin, IAA production, HCN production	Haas et al., 1991
Pseudomonas fluorescence Pf-5 (Pf-5)	Cotton rhizosphere	Antibiotic production including pyrrolnitrin, pyoluteorin and DAPG Siderophores pyochelin and pyoverdine HCN production	Howell and Stipanovic. 1979
Pseudomonas sp. G16 (PG16)	Groundnut rhizosphere	Siderophore production	Chaubey, 2015, Patel and Archana, 2018
Pseudomonas sp. G19 (PG19)		Antifungal activity, phosphate solubilization	
Pseudomonas sp. G22 (PG22)		Antifungal activity, siderophore production	
Pseudomonas sp. G38 (PG38)		Siderophore production	
Rhizobium sp.	Pigeon pea nodule	Nodulating strain for *Cajanus*	Geetha et al.,

ST1 (RST1)	(Geetha et al., 2009)	*cajan*	2009
Rhizobium sp. IC3109 (RIC3109)	Pigeon pea nodule (IARI, New Delhi, India)	Nodulating strain for *Cajanus cajan*	This Study
Sinorhizobium fredii NGR234 (NGR234)	Lablab bean	Bio-inoculant strain for a wide range of cereal crops.	Saldaña et al., 2003
Other strains	**Source (Ref)**	**Description**	**Reference**
Staphylococcus aureus	Department culture collection. (Department of Microbiology, MS University, Vadodara, Gujarat, India)	Sensitive towards pseudomonas antibiotic 2, 4-DAPG	Cesa-Luna et al., 2020
Escherichia coli DH5-α (DH5α)		High transformation efficiency	-
Rhizoctonia solani		Soil-borne fungal pathogen against broad range cereal-legume crops.	
Fusarium udum	Pulse Research centre (AAU, Vadodara, Gujarat)	Soil born fungal pathogen, a major biotic factor adversely affecting pigeon pea crops.	Dukare & Paul, 2021

2.2.2. Molecular identification of isolates

Pseudomonad isolates PG16, PG19, PG38 and *Rhizobium* sp. IC3109 were sequenced for strain identification. Universal eubacterial primers 27F and 1107R (Chaturvedi and Archana, 2012), were used for the amplification of 16S rRNA partial gene using the genomic DNA from pure cultures as template. PCR reactions were carried out in 30µl reaction mixtures consisting 10X Taq DNA polymerase buffer (with $MgCl_2$), 1µl dNTP (10mM stock), 2.5µl of each of the primers (10µM stock), 0.8µl of Taq DNA polymerase (5U) and 10ng template. Amplification was carried out in thermal cycler (Applied Biosystems, USA) with an initial denaturation at 94°C for 3min, followed by 30 cycles, each consisting of denaturation at 94°C for 45s, annealing at 58°C for 45s, elongation at 72°C for 1min with a final elongation at 72°C for 10min. Amplicons were detected by electrophoresis on 1.8% (w/v) agarose gels in TAE buffer with ethidium bromide (EtBr) as the indicator. The purified amplification product was cut out from the agarose gels, and extracted using glass wool method and re-concentrated

by phenol-chloroform method. The samples were sent for the 16S rRNA partial gene sequencing analysis by the Sanger's method (AgriGenome Labs Pvt Ltd, Kochi, Kerala). The best read from the chromatogram was converted to FASTA format and NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/) analysis as well as RDP-II project database SeqMatch (http://rdp.cme.msu.edu/seqmatch/) were performed for best match.

2.2.3. Preparation of bacterial inoculum

The bacterial inocula were prepared by growing the strains in broth medium to be used for the experiment. The cells were washed twice using normal saline (0.85% NaCl w/v) and resuspended in the same.

2.2.4. Compatibility among the strains for consortia preparation

Since the pseudomonads are known to produce antibacterial molecules, therefore compatibility among the bacterial strains was determined by spread plating the rhizobia and EC1D onto LB agar plates, the pseudomonad strains were spot inoculated on it and the plates were incubated for 24h in the inverted position. Bacterial incompatibility was determined based on the development of clear zones around the pseudomonad colonies, while absence of clear zone formation would indicate no growth inhibition and synergy among the strains under study. *Staphylococcus aureus* was used as positive control since it is known to be sensitive towards pyocyanin and HCN.

2.2.5. Primary screening for salt stress tolerance of bacteria under study

The bacteria were inoculated in LB broth, supplemented with salt concentrations ranging from 0 - 5% NaCl (w/v), and grown under shaking condition for 48h. LB medium was found to be sub-optimal for the growth of NGR234, therefore salt stress tolerance was assessed in TY broth. Bacterial growth was estimated by collecting samples at 2h intervals by optical density measurements conducted using Shimadzu (UV-1601) UV-Vis spectrophotometer at 600nm.

The PGPR traits and growth curves were conducted in three independent set of experiments and results are expressed as mean ± standard deviation.

2.2.6. Effect of salinity stress on plant growth promoting traits of bacteria

The bacterial strains were assessed for the following PGPR traits conducted under increasing saline conditions. All the following experiments were performed in triplicates, while salt stress was amended in the growth medium in the concentrations ranging from 0 – 5% NaCl

(w/v) before sterilization. To minimize the effect of any other additional stress factor besides salinity, the bacterial inoculum was prepared in the same growth medium, used for test experiment. Strains were grown overnight on a rotary shaker, separated by centrifugation, washed (twice) with 8.5 g L^{-1} NaCl (normal saline) solution and resuspended in an equal volume of same to be used for inoculation. Bacterial inoculum was added to the growth medium (10^8 cfu) and incubated in shaking conditions at 30°C.

2.2.6.1. IAA production

Bacteria were grown in M9 minimal medium (Appendix I) amended with L-Tryptophan (1mg/L). The experiment was conducted up to 7d and aliquots were collected at 3 and 7d interval and the cell-free supernatants were quantified for the phytohormone production by the Salkowski's method (Patel and Archana, 2017) in 96 well micro-titre plates, using pure IAA as standard for reference at OD_{530nm}, development of pink colour indicated IAA production.

2.2.6.2. Siderophore production

Siderophore production was estimated by the universal method of Schwyn and Neilands (1987). Bacterial ability to produce siderophores in iron-deficient conditions was assessed in Chrome azurol S (CAS) agar plates (Appendix I). Glassware soaked in 5% H_2SO_4 were used for media preparation, M9 broth was prepared in double distilled water and deferrated using 2% 8- hydroxyquinoline in chloroform to remove all traces of iron. A limited concentration of $FeCl_3$ (1mmol/L) was added to the medium to initiate bacterial growth before iron starvation could begin.

Bacterial strains were inoculated in deferrated M9 medium and kept on shaker overnight. 10µl bacterial cultures were spot inoculated on CAS agar plates, development of orange-yellow zone around the colonies indicated siderophore production.

Quantification of siderophore production was performed in broth medium, the experiments were conducted for 24h and the siderophore content was measured by CAS-Shuttle assay as described (Dutta and Thakur, 2017). The culture supernatant was mixed with equal volume of CAS reagent in 96 well microtiter plates. An uninoculated control set was used as reference, after 30min incubation, the plates were read spectrophotometrically at 630 nm. Siderophore content was calculated by using following formula:

$$\% \text{ Siderophore units} = \frac{Ar - As}{Ar} \times 100 \qquad \text{Eq. (1)}$$

Where Ar = Absorbance of reference at 630nm (CAS reagent)

As = Absorbance of sample at 630nm.

The data analysis was performed by using siderophore production by EC1D in no salt control as 100%. Significant differences in the mean values were assessed by one-way ANOVA ($P \leq 0.05$).

2.2.6.3. Biofilm formation

Biofilm formation was estimated as given by Zupančič et al. (2018). Luria broth diluted to $1/5^{th}$ strength was used as the growth medium for biofilm estimation using 24 well polystyrene plates. 2ml of sterile broth was added aseptically followed by bacterial inoculation, the plates were sealed with the help of parafilm and kept for incubation till 48h on shaker. After incubation, 100µl of 0.1% Crystal Violet was added to each well, 10min incubation at room temperature (RT), wells were flooded sterile phosphate buffered saline (PBS) thrice to remove the excess stain and un-adhered cells from the walls and the plates were air-dried. Crystal violet (CV) bound to the biofilm was extracted later using methanol and measured spectrophotometrically at OD_{595nm}. The degree of biofilm formation, was indicated by the intensity of CV. Controls was performed with Crystal Violet binding to the wells exposed to sterile growth medium.

2.2.6.4. Phosphate solubilization

Overnight grown cultures in M9 minimal medium (MM9) medium (Appendix I), were spot inoculated on Pikovskaya's (Hi-Media, India) for mineral phosphate solubilization (MPS) activity, and kept for 48h (Sharaff et al., 2017). Formation of a clear halo/zone around the growth of each culture indicated organic acid production and solubilization of the inorganic tricalcium phosphate present in the medium. P-solubilization index was measured as given below (Gupta and Pandey, 2019), where halozone diameter was measured including microbial colonies.

$$\textit{P-solubilization Index (PSI)} = \frac{Halozone\ diameter}{colony\ diameter} \qquad \textit{Eq. (2)}$$

2.2.6.5. HCN production

The hydrogen cyanide (HCN) production was assessed as described by (Y. Singh et al., 2013). Nutrient agar (NA) plates amended with 4.4 g/L of glycine were spot inoculated with 20µl of test culture respectively. Whatman filter paper No. 1, dipped in Picric acid solution (Appendix I) were placed on the petri plate lid and the plates were sealed using parafilm.

Plates were incubation in upright position for 4d. Change in colouration of filter paper from yellow to reddish brown indicated HCN production.

2.2.6.6. Ammonia Production

Ammonia production was detected using Nesseler's reagent (Appendix I) (Sadat, et. al., 2012). It is a qualitative test where, the Nesseler's reagent (K_2HgI_4) reacts with ammonia, under strongly alkaline conditions to produce an orange-yellow coloured precipitate. Overnight grown cultures were inoculated in 10 ml peptone water and incubated for 48h. Equal volume of cell-free supernatant was mixed with Nesseler's reagent and incubated for 30min. Development of brown to yellow colour indicated a positive test for ammonia production. The intensity of the colour produced is directly proportionate to the ammonia concentration.

2.2.7. Antifungal activity

Pseudomonas strains were grown overnight in pseudomonas broth with different salt concentrations, cell-free supernatant was filter sterilized and 100µl sample was added to wells (6mm diameter; 1cm from the edge) bored in fresh PDA plates (punched using a cork-borer). Fungal plug ($1cm^2$ size) cut out from the edges of a fully developed fungal colony of *Fusarium* sp. and *R. solani* were placed in the center of these PDA plates and allowed to incubate until the fungal colony reached the edge of plates. A zone of inhibition around the wells indicated production of antifungal metabolites in the strains. Similar set up was done using uninoculated bacterial growth medium using respective salt concentrations as a negative control.

2.2.8. Consortium preparation

Three-member consortia were prepared by mixing one strain each from *Enterobacter* sp., *Pseudomonas* spp. and rhizobia in equal proportions (1:1:1 ratio) such that the initial cell count in individual and consortia experiment, were similar (10^8 cfu/ml). Therefore, each consortium was made up with one third amount of individual strains, and the resultant PGPR trait values were assessed in comparison with individual strains for synergistic effect of bacteria when combined together. A total of 18 consortia were prepared with all possible combinations for further studies of PGP traits and the effect of consortia development on the salt stress tolerance of microbes and their ability to demonstrate these traits in salt conditions (Figure 2.1). All the bacterial consortia were assessed for above-mentioned PGPR traits under

saline stress in the concentrations 1 – 5% NaCl (w/v), while no salt treatment was used as a control set.

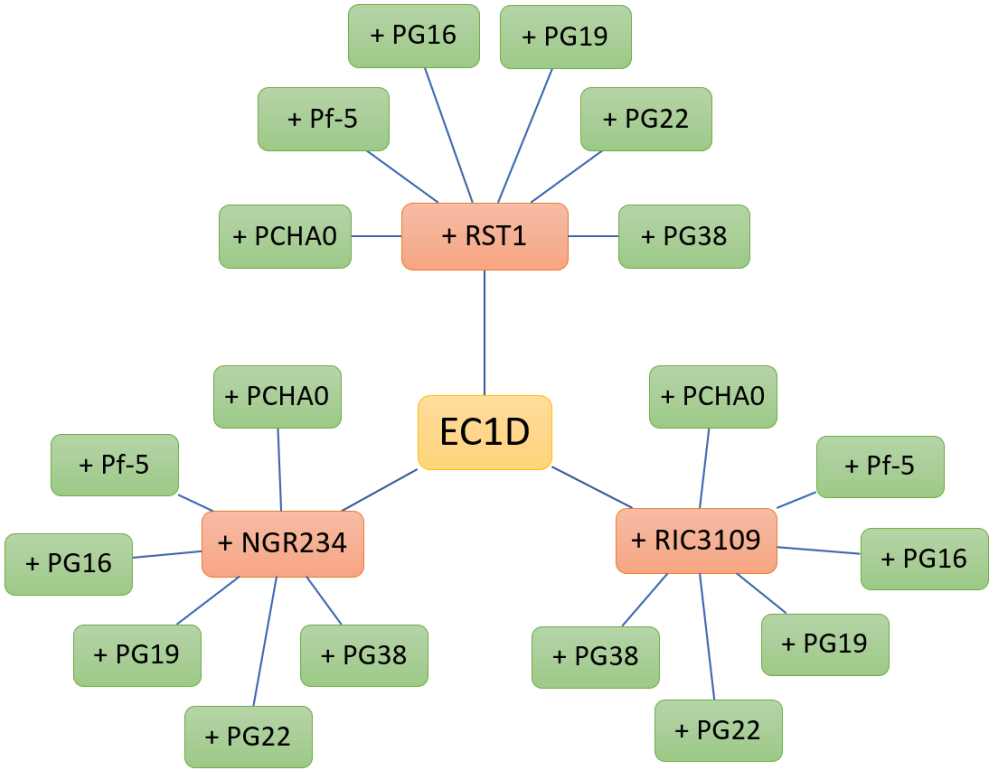

Figure 2.1: Preparation of consortia using one strain from each group of PGPR. Enterobacter sp. is represented in yellow, the rhizobial strains are represented in red colour while the ones in green represent pseudomonad strains.

2.2.9. Characterization of siderophores

Bacterial cultures were grown overnight in 100 ml deferrated LB broth amended with $FeCl_3$ for siderophore production which were characterized using thin layer chromatography (TLC) technique (Hussein and Joo 2017). Siderophores were extracted twice in ethyl acetate using a separating funnel. The resultant solvent was concentrated by evaporation, in a boiling water bath in fume hood, and re-dissolved in 5ml Ddw and stored at 4˚C. The crude extracts were spotted on TLC silica gel plates (12cm x 12cm; Merck, India), 20µl volume from each extract. The plates were kept in a tertiary mixture mobile phase containing n-butanol, acetic acid and Ddw (3:1:1 ratio) and allowed to run till 8cm distance. The dried plates were observed under UV light for identification of siderophore spots and later on developed by spraying with 0.1M $FeCl_3$ in 0.1N HCl. Development of grey spots indicated catecholate type while reddish-purple coloured spots indicated hydroximate siderophores.

2.2.10. Consortial synergy under iron-deficient conditions

For further understanding of the bacterial synergy in a consortium, siderophore cross-utilization studies were performed. The siderophore extracts from bacterial strains were placed in wells and growth of individual bacteria in iron-starved medium was checked. For this first the minimum EDTA concentration inhibitory for each strain was determined as follows.

EDTA MIC: Deferrated (Appendix I) M9 agar medium was used as the growth medium. Sterile EDTA (pH 8.0) at concentrations ranging from 1mM to 15mM was added to the growth medium and the bacteria were streaked as lines to determine the minimal inhibitory concentrations (MIC) at which native siderophores produced the bacteria were rendered incapable of chelating iron and making it available to cells for survival.

Siderophore cross-Utilization: The test bacteria were spread plated onto the M9 agar amended with the respective EDTA-MIC. The agar medium was perforated to create wells at 1.5cm distance from the edge of the plates. The siderophores extracted (Section 2.2.9.) from each bacterial strain were added to the wells (10µl) and allowed for diffusion at 4˚C for 30min after which the plates were incubated in inverted positions for 24-48h period to develop zone of exhibition. $FeSO_4$ (100 µM) was used as a positive control and sterile N-saline was used as negative control.

2.2.11. Statistical analysis

The significant differences among the growth attributes of control sets and the PGPR treated plants under no salt and salt stressed conditions were analysed by the One-way ANOVA ($P \leq 0.05$). The values were calculated as mean ± standard deviation of the triplicate sets.

2.3. Results

2.3.1. Identification of bacterial isolates based on 16S rRNA sequencing

Since 16S rDNA sequencing was not previously reported for the isolates PG16, PG19, PG38 and RIC3109, these were subjected to molecular identification in this study for confirmation of them as *Pseudomonas* spp. and *Rhizobium* spp. The pseudomonad isolates were identified having similarities with *P. putida*, *P. protegens* and *P. fluorescence* (Table 2.2) and were thus confirmed to be *Pseudomonas* spp, PG38 showed very high similarity to standard strains PCHA0 and Pf-5 used in the present study. Fluorescence was commonly observed in all the *Pseudomonas* isolates under study. All the matching strains have been reported as PGPR by

other researchers. Strain IC3109, the pigeon pea nodulating isolate obtained from IARI, New Delhi was found to match with *Rhizobium* spp. and phylogenetically related *Agrobacterium tumefaciens*. The 16S rRNA partial sequences generated are deposited in the NCBI GenBank database and accession numbers of the same are as given in Table 2.2.

Table 2.2: Identification of pseudomonad isolates based on 16S partial sequencing.

Bacterial Strain (GenBank Accn. No,)	Closest match (% Similarity)	PGPR activity reported in similar strains	Reference
PG16 **(MZ557397)**	*Pseudomonas plecoglossicida* (98.55%)	Antioxidant & biofilm, hydrolytic enzymes production.	Rahmoune et al., 2017
	Pseudomonas putida (98.46%)	Plant growth promotion, biosurfactant activity.	Mishra et al., 2020
	Pseudomonas fluorescence (98.46%)	Enhanced plant growth and salt stress amelioration.	Nawaz et al., 2020
PG19 **(MZ557800)**	*Pseudomonas putida* (98.77%)	Production of diverse antimetabolite compounds, biocontrol activity.	Agisha et al., 2019
	Pseudomonas plecoglossicida (98.77%)	Plant growth promotion, auxin synthesis and phytoremediation.	Bakaeva et al., 2020
	Pseudomonas montelli (98.77%)	Plant growth promotion and biocontrol activity under drought stress.	Sandhya et al., 2017
PG38 **(MW234000)**	*P. protegens* CHA0 (97%)	Bioactive metabolites, 2, 4-DAPG production, HCN and IAA production.	Chaubey et al., 2015
	P. protegens Pf-5 (97%)	Antibiotic production, HCN production	Patel & Archana, 2018
RIC3109 **(MW040081)**	*R. pusense* (99.89%)	IAA, ACCd, siderophore and ammonia production	Chaudhary et al., 2021
	Agrobacterium tumefaciens (99.89%)	Increased N and P uptake in plants.	Ejaz et al., 2020

2.3.2. Consortial compatibility among bacterial strains

All combinations of the bacterial strains used were found to be compatible as they did not develop any zone of inhibition among the strains (data not shown). However, *S. aureus* was inhibited by all the pseudomonad strains.

2.3.3. Salt Tolerance of PGPR strains

The bacteria showed varying levels of salt stress and it was observed that each group of bacteria had similar levels of salinity tolerance (Figure 2.2). Maximum salt tolerance was observed in EC1D (5%), followed by the pseudomonads (4%) and the least in rhizobia (2%). NGR234 was least tolerant towards salt stress (1%) but was able to survive at 2% salinity.

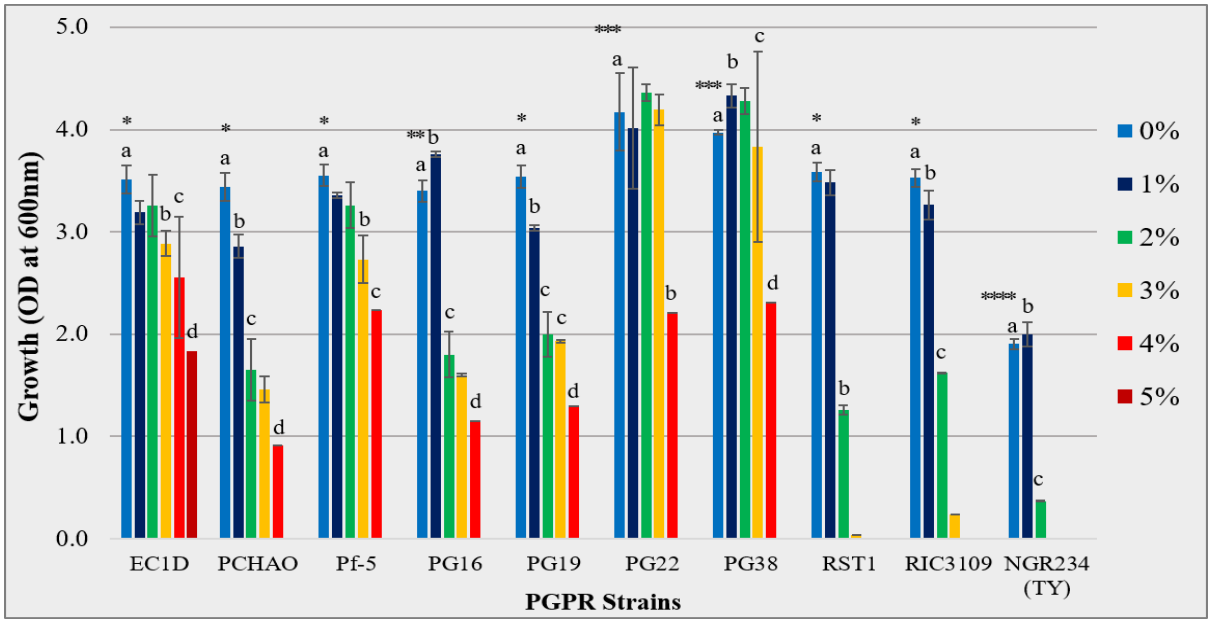

Figure 2.2:Growth of PGPR strains under salt stress. The Asterisk (*) denotes significant difference in growth among strains without salt (P≤0.05), Different letters represent significant variation in the growth of strains under increasing NaCl concentration for each strain (P≤0.05).

Negative impact of increasing salinity on bacterial growth was observed in all strains except for PG22 and PG38, which showed similar growth (density) at high salt stress (up to 3% NaCl) compared to 0% control. Followed by these, growth in EC1D and Pf-5 was also less severely affected by the increasing salinity. At 1% salinity, most of the bacterial strains showed growth comparative to the no salt control. Only EC1D was able to survive at 5% NaCl.

2.3.4. PGPR activity under salt stress – Individual strains vs Consortia

The results of the PGPR traits were analyzed as a comparison between individual strains and their consortia to evaluate the effect of consortia formation on the PGPR traits in control as

well as salt stressed conditions. It was observed that the salt concentrations inhibiting bacterial growth did not define minimal inhibitory concentration (MIC) for trait production.

2.3.4.1. IAA Production

IAA production was the highest among rhizobia and EC1D but the concentration varied with incubation period. While it was progressive with prolonged incubation in EC1D (maximum at 7d, 42µg/ml). In *Rhizobium* spp., IAA production was maximum on 3d which decreased from ~28µg/ml to ~16µg/ml on 7d. As shown in Figure 2.3, pseudomonads and NGR234 exhibit low IAA production (~5µg/ml and 5.5µg/ml respectively) but it was significantly similar at all salt concentration up to 2% salinity.

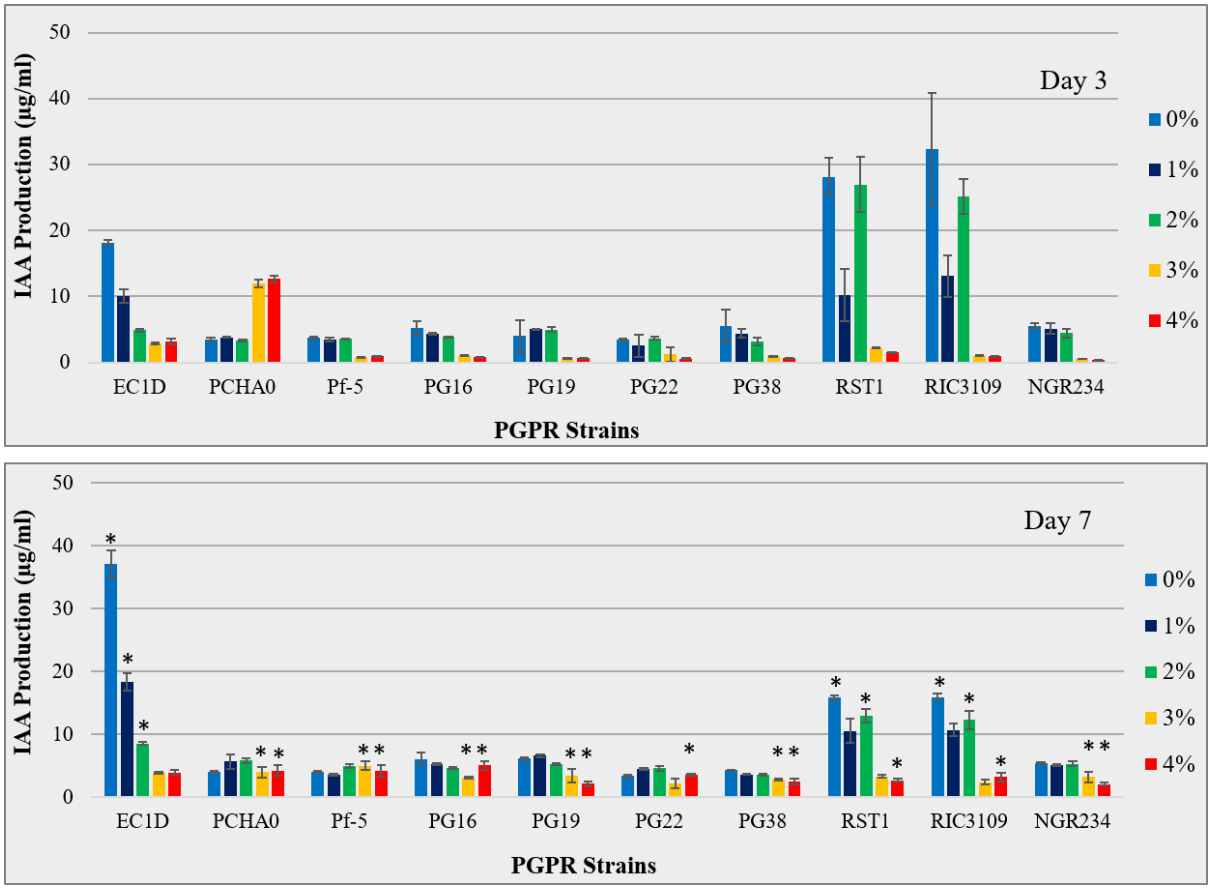

Figure 2.3: IAA production in individual PGPR strains under salt stress at 3d and 7d. The Asterisk (*) represents significant difference with increased incubation period at each salt concentration. The significance was calculated based on ANOVA: Single factor analysis (P≤0.05).

With the exception of PCHA0, delayed phytohormone production was observed at 3% and 4% salinity, similar to EC1D and maximum IAA concentration was observed at the end of 7d. IAA production at 3% and 4% NaCl in PCHA0, was highest (~12µg/ml) on 3d. No IAA production was observed beyond 4% salinity.

In *consortia*, the overall IAA production was reduced in comparison to EC1D and *Rhizobium*. A notable improvement observed was an ~2-fold increase in the IAA produced in consortia at 3% and 4% salinity, except those with NGR234. Most importantly, it was observed that pseudomonads and rhizobia each showed strain specific influence on the trait outcome (Figure 2.4). As observed in individual strains, IAA production in consortia with PCHA0, was significantly reduced after 3d incubation among the consortia as well. Among rhizobia, stability in IAA concentration was observed at all salt concentrations in NGR234 and its consortia also showed similar property.

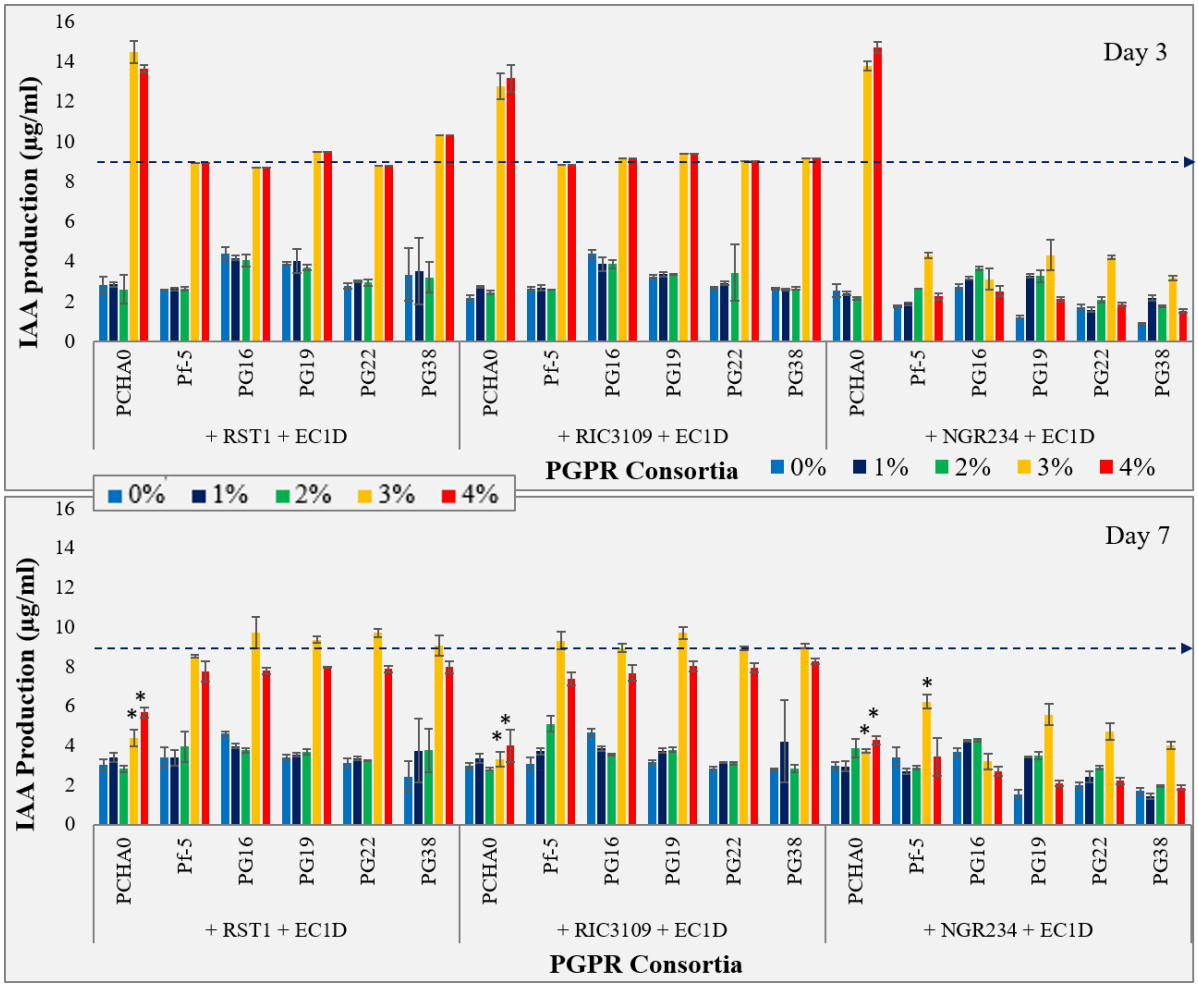

Figure 2.4: IAA production in PGPR consortia under salt stress. The dotted line represents an average concentration of IAA observed on day 3, which was reduced in all the consortia at day 7. The Asterisk (*) indicated significant variation in the phytohormone production with increased period of incubation as determined by ANOVA: Single factor analysis (P≤0.05).

After a 7d period of incubation - the consortia, without PCHA0 and NGR234, showed an equivalent range of IAA production, depending on salinity stress. The IAA production in consortia at 1% and 2% NaCl were similar to no salt controls throughout the period of incubation.

2.3.4.2. Siderophore production

Siderophore production showed a trend of decline with increasing salt. The phenotype was completely inhibited at 3% salinity while the bacterial strains had shown tolerance towards higher salt concentrations ie., for EC1D and the pseudomonads. On the CAS agar plate, with the exception of rhizobial strain NGR234, all the strains showed positive siderophore production.

Quantitative analysis in broth medium revealed siderophore production in NGR234 as well. Maximum siderophore production under salt stress was observed in EC1D (82.48%), being the only strain that was least affected till 3% salinity with siderophore concentration similar (11.8% reduction) to no salt control. The siderophore production in EC1D in no salt control was used as reference where as shown in Figure 2.5, the columns indicate the percentage difference in siderophores produced among the bacterial strains with respect to EC1D, the letters above them indicate significant variation among bacterial strains in no salt condition determined using ANOVA single factor analysis ($P \leq 0.05$) and the Asterisk (*) indicates significant variance in the siderophores produced by the same bacteria but under increasing salt stress.

Pf-5 produced significantly equivalent concentration of siderophores as EC1D in normal condition. It was closely followed by PG38 in 0% and 1% salt stress, PG16 from 0% to 2% salt stress. Most of the strains produced siderophores up to 2% NaCl except Pf-5 and NGR234 where it was negligible. NGR234 was the only strain producing significantly higher siderophores at 1% NaCl as compared to no salt control.

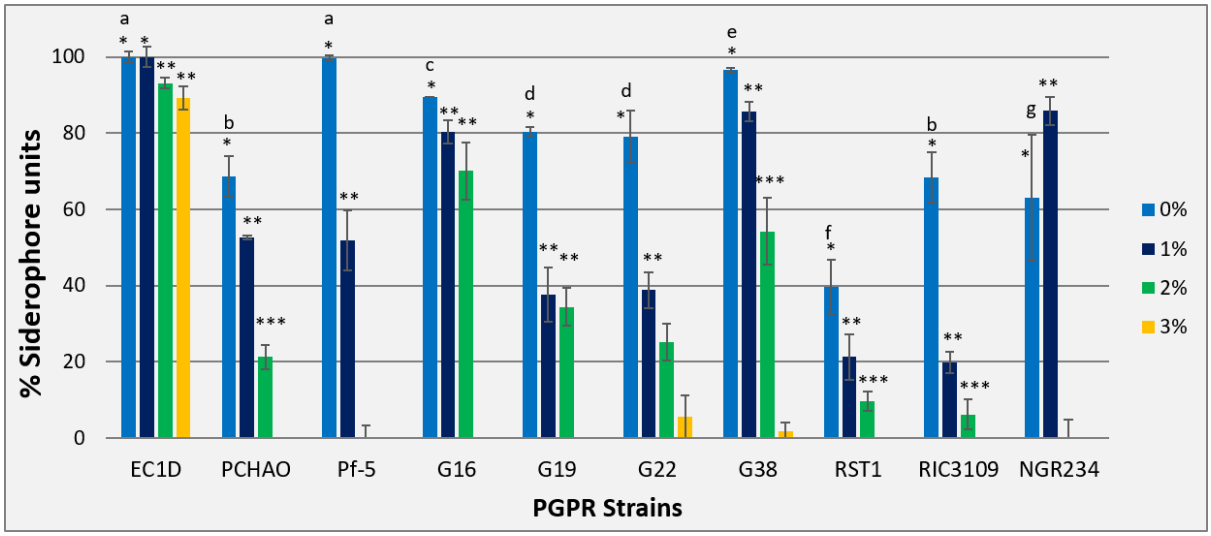

Figure 2.5: Siderophore production by individual PGPR strains under salt stress with respect to EC1D in no salt control. The Asterisk (*) represents significant difference among the strains at different salt concentrations, while the letters indicate significant variation among the strains in no salt condition, done by ANOVA: Single factor analysis (P≤0.05).

In *consortia*, the overall siderophore production was less in comparison to most of the individual strains, but all the combinations produced more than 60% siderophore units till 2% NaCl concentration, which was also equivalent to no salt control (Figure 2.6). No siderophores were produced beyond 3% salt stress. Since EC1D was a common factorial in all the combinations, it was observed that siderophore activity in consortium was dependent on the pseudomonad member. The siderophore production in consortia with Pf-5 and PG19 was significantly higher than others.

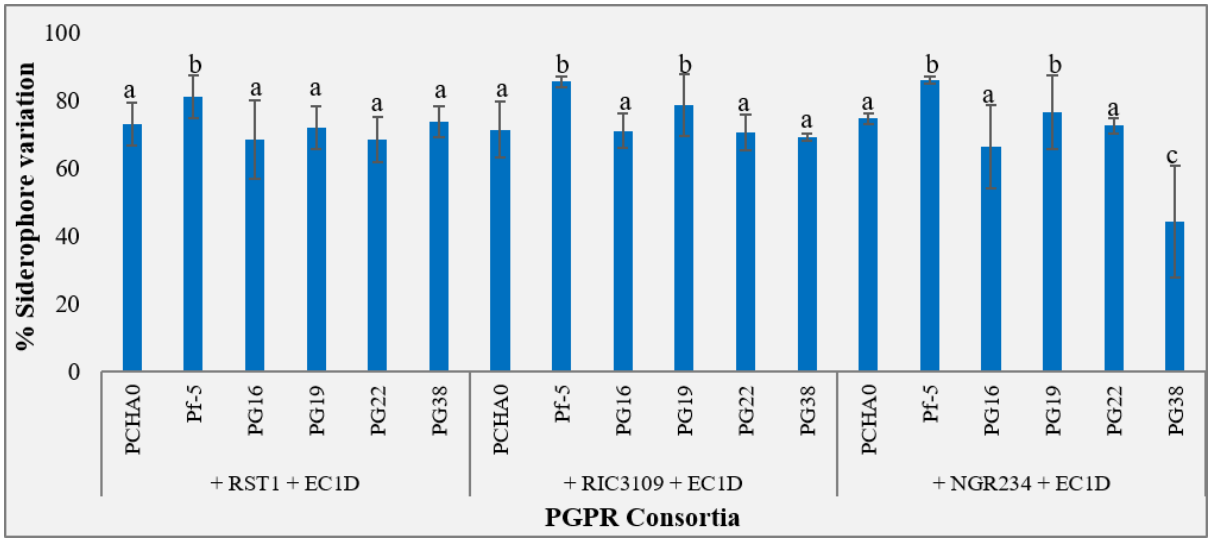

Figure 2.6: Siderophore production in bacterial consortia under no salt condition. The letters indication significant variation for siderophore production among the consortia in no salt condition.

Consortia having PG19, PG22 and PG38 produced siderophores at 3% salinity as well which was significantly higher compared to other consortia. The consortia PG38 and NGR234

produced lowest amount of siderophores in no salt condition (Figure 2.7), however, an increase in siderophore production with increasing salinity was observed in NGR234 which was ~1.5-folds higher at 3% salinity compared to control and there was no significant difference in all the salt concentrations.

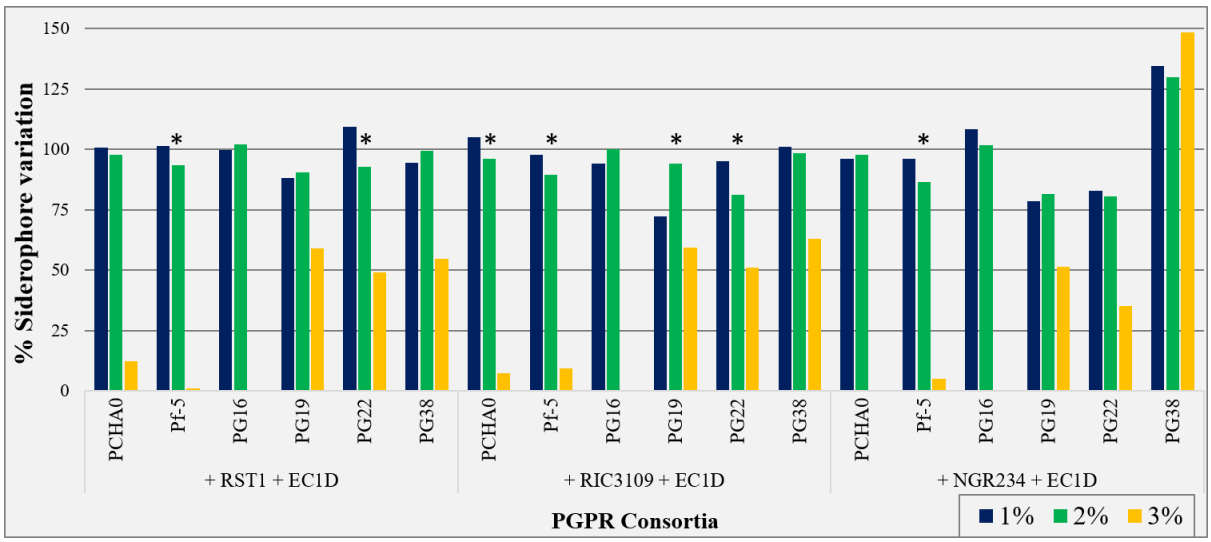

Figure 2.7:Percent variation in siderophore production under salt stress by the PGPR consortia, with reference to siderophore production in no salt control respectively. The Asterisk (*) indicates significant difference in siderophore production by the consortia under different salt concentrations, done by ANOVA: Single factor analysis (P≤0.05). The values obtained at 3% salinity have not been marked since they were significantly different in all the combinations with the exception on C3N.

2.3.4.2.1. Identification of siderophore types

TLC plate assay revealed 2 types of siderophores among the strains under study. The pseudomonads showed hydroxamate type of siderophores while the other strains including EC1D and rhizobia had catecholate type of siderophores.

2.3.4.2.2. Siderophore Cross Utilization Assay

Cross utilization of siderophores for survival on EDTA (MIC) amended medium was confirmed based on the zone of exhibition developed around the externally provided siderophores (Figure 2.8).

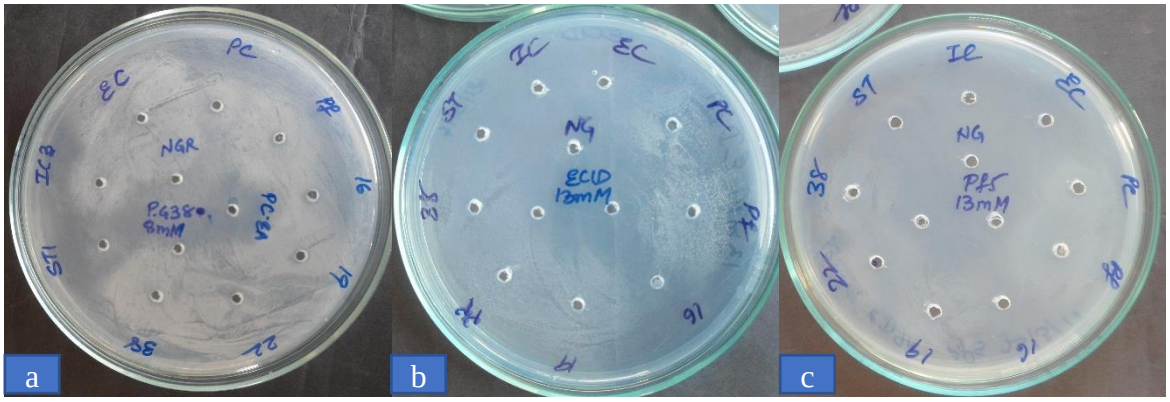

Figure 2.8: Zone of exhibition on EDTA plates indicating cross utilization of siderophores in three strains a) PG38, b) EC1D and c) Pf-5.

The strains EC1D, Pf-5, PG19, PG22, PG38 and RST1 have shown maximum growth in presence of siderophores obtained from other PGPR's, as well as exhibit a broad range for utilization of non-native siderophores (Table 2.3). RIC3109 was unable to utilize siderophores from other strains. While PG16 was able to cross-utilize siderophores from EC1D only, PG16 siderophores were taken up by Pf-5 strain only. These results corelate to the siderophore production observed in the consortia at 2% salt stress showing consortial synergy. PG16 produced high amount of siderophores, but its siderophores were not utilized by other strains except pseudomonads, therefore, its consortia were also not able to produce siderophores at 2% salinity. Similarly, Pf-5 did not produce siderophores at 2% salinity, even though rhizobia produced siderophores at this concentration, the consortia having Pf-5 showed negligible amounts of siderophore production. But the absence of siderophores in NGR234 at 2% salt concentration was overcome by the pseudomonad strains indicating siderophore production in consortia was highly dependent on the pseudomonad strain present.

Table 2.3: Siderophore cross utilization among PGPR strains.

	PGPR Strains (Zone of exhibition in mm)									
Siderophore extracts	EC1D	PCHAO	Pf-5	PG16	PG19	PG22	PG38	RST1	RIC3109	NGR234
EC1D	16	-	13.5	20.6	16	20	26.5	16.6	-	-
PCHA0	21	15	19		15.3	21	12.5	18	-	-
Pf-5	21	16.6	24.6	-	19.3	22.5	19	19.6	-	10
PG16	-	-	17.3	15	-	-	-	-	-	-
PG19	23	18.5	18	-	23	19	19.5	14.3	-	-
PG22	24	20.6	22.6	-	18	22.5	16.5	19.3	-	-
PG38	23.6	17.6	22	-	15.7	22.5	22.5	12.5	-	12
RST1	16.5	18	19	-	13	20	11.5	22.3	-	-
RIC3109	18.3	-	18	-	14.3	20	15.5	-	-	-
NGR234	-	-	-	-	-	-	-	-	-	-

2.3.4.3. Biofilm production

All the strains were able to produce biofilm although the difference was independent of the PGPR group to which a strain belonged. The maximum biofilm production was observed in PG38, followed by PG22, PG16 and Pf-5. Biofilm production in the rhizobial strains was least among the strains under study. Except for PG22 and RST1, salt stress stimulated an increased biofilm production in the bacteria. Biofilm production was limited beyond 3%

salinity. The percentage change in biofilm production due to salt stress was as described in the Table 2.4. The values indicated in green represent ~1.5-fold change in the biofilm production, while those in red represent greater than 2-fold increase in biofilm production due to salt stress. The highest biofilm production under salt stress was observed in PG38 at 2% NaCl concentration.

Table 2.4: Biofilm production by individual PGPR strains and Percentage variation in biofilm production under salt stress. The numbers indicated in red under no salt control are for highest biofilm production, while those under salt stress in green indicate ~1.5-fold or greater and numbers in red 3-fold increase or higher in the biofilm production.

PGPR strains	Biofilm production	% change under salt stress		
	No salt control	1%	2%	3%
EC1D	0.202	87	175	232
PCHA0	0.221	**148**	110	113
Pf-5	0.430	**289**	**301**	**296**
PG16	**0.724**	**168**	172	49
PG19	0.269	**287**	**377**	**406**
PG22	**1.090**	63	72	71
PG38	**1.331**	**259**	**292**	**184**
RST1	0.085	98	96	88
RIC3109	0.082	**294**	126	139
NGR234	0.111	111	134	**247**

In consortia, the biofilm production was significantly higher than most of the individual strains. All the consortia with Pf-5, PG22, PG38, RST1 and RIC3109 produced highest biofilm among all combinations. While in salt stress the biofilm production increased in most of the consortia, it was highest at 2% salinity (Table 2.5).

Table 2.5: Biofilm production by consortia and percentage variation in biofilm production under salt stress. . The numbers indicated in red under no salt control are for highest biofilm production, while those under salt stress in green indicate ~1.5-fold or greater and numbers in red 3-fold increase or higher in the biofilm production.

PGPR Consortia		**Biofilm production**	**% change under salt stress**		
		No salt control	**1%**	**2%**	**3%**
EC1D + RST1	PCHA0	0.824	24.5	67.4	88.8
	Pf-5	**1.437**	**160.4**	**149.9**	**145.4**
	PG16	0.337	109.2	99.1	78.5
	PG19	0.378	**163.4**	**234.2**	**222.6**
	PG22	**1.331**	128.7	123.8	130.0
	PG38	**0.972**	**241.3**	**256.7**	**212.3**

EC1D + RIC3109	PCHA0	0.314	**159.9**	**176.9**	**146.0**
	Pf-5	**1.644**	**162.2**	**153.1**	121.8
	PG16	0.276	120.8	94.4	72.4
	PG19	0.488	136.4	**255.4**	**216.8**
	PG22	0.681	127.7	**276.7**	**303.3**
	PG38	**0.942**	**247.3**	**311.3**	**196.3**
EC1D + NGR234	PCHA0	0.236	144.4	**166.2**	113.4
	Pf-5	0.697	43.3	**234.4**	151.7
	PG16	0.257	**243.5**	**231.2**	**236.1**
	PG19	0.111	106.3	**179.0**	138.7
	PG22	0.434	46.1	109.1	**281.0**
	PG38	0.611	88.0	**208.9**	**183.3**

2.3.4.4. Phosphate solubilization activity

On Pikovskaya's agar, salt stress inversely affected the P-solubilization efficiency, with a few exceptions. The PSI data was divided into 3 categories based on the halozone diameter to colony diameter. The strains were considered as good solubilizers if the ratio exceeded 1.5 folds, while those showing halozone but less than 1.5-folds were considered as poor solubilizers. In some, only the bacterial growth was observed without halozone formation, these were considered as P-deficiency tolerant strains (Table 2.6).

Among individual strains, EC1D and the pseudomonads Pf-5, PG22 and PG38 showed maximum PSI activity in the same order. PG16, PG19 and RST1 did not produce a clear zone but were able to grow on the P-deficient Pikovskaya's medium under salt stress. While NGR234 produced a clear halo on Pikovskaya's medium, it could not grow in salt stress and RIC3109 did not grow at all on the p-deficient medium.

Table 2.6: Phosphate solubilization by the PGPR strains and their consortia under increasing concentration of salt stress.

Salt (%)→		0%	1%	2%	3%	4%	5%
EC1D		2.14 ± 0.2	1.67 ± 0.7	1.67 ± 0.7	1.50 ± 0.7	1.00 ± 0.5	0.00
PCHA0		1.50 ± 0.5	1.33 ± 0.6	0.00	0.00	0.00	0.00
Pf-5		1.67 ± 1.0	1.57 ± 0.8	1.33 ± 0.7	1.33 ± 0.5	1.00 ± 0.5	1.00 ± 0.5
PG16		1.00 ± 0.5	1.00 ± 0.5	0.00	0.00	0.00	0.00
PG19		1.00 ± 0.5	1.00 ± 0.5	0.00	0.00	0.00	0.00
PG22		1.75 ± 1.0	1.50 ± 0.1	1.33 ± 0.6	1.00 ± 0.5	1.00 ± 0.5	1.00 ± 0.5
PG38		1.67 ± 0.9	1.29 ±	1.33 ± 0.6	1.00 ± 0.5	1.00 ± 0.5	1.17 ± 0.5
RST1		1.00 ± 0.5	1.00 ± 0.5	1.00 ± 0.5	0.00	0.00	0.00
RIC3109		0.00	0.00	0.00	0.00	0.00	0.00
NGR234		1.17 ± 0.8	0.00	0.00	0.00	0.00	0.00
EC1D + RST1	PCHA0	1.67 ± 0.8	1.83 ± 0.6	1.60 ± 0.7	1.00 ± 0.9	1.00 ± 1.0	0.00 ± 0.6
	Pf-5	1.67 ± 1.0	1.50 ± 0.5	1.29 ± 0.5	1.17 ± 0.5	1.29 ± 1.0	1.17 ± 0.5
	PG16	2.00 ± 0.8	1.80 ± 0.8	2.00 ± 0.6	1.00 ± 0.6	1.00 ± 1.0	1.00 ± 0.7
	PG19	1.67 ± 0.9	1.17 ± 0.8	1.17 ± 0.4	1.17 ± 0.7	1.00 ± 0.8	1.00 ± 0.6
	PG22	1.43 ± 0.5	1.29 ± 0.5	1.29 ± 0.8	1.14 ± 0.5	1.14 ± 1.18	1.14 ± 0.7
	PG38	1.43 ± 1.0	1.29 ± 0.8	1.33 ± 0.8	1.17 ± 1.0	1.14 ± 0.9	1.00 ± 0.6
EC1D + RIC3109	PCHA0	1.50 ± 0.7	1.43 ± 0.6	1.33 ± 0.4	1.00 ± 1.0	1.00 ± 1.0	1.00 ± 0.4
	Pf-5	1.25 ± 1.0	1.33 ± 1.0	1.29 ± 0.5	1.14 ± 0.5	1.17 ± 0.8	1.17 ± 0.6
	PG16	1.67 ± 0.7	1.60 ± 0.5	1.60 ± 0.6	1.00 ± 1.0	1.00 ± 1.0	1.00 ± 0.6
	PG19	1.67 ± 0.8	1.33 ± 0.4	1.20 ± 0.8	1.17 ± 0.5	1.00 ± 1.0	1.00 ± 0.4
	PG22	1.67 ± 0.7	1.29 ± 0.5	1.29 ± 1.0	1.17 ± 0.6	1.14 ± 0.5	1.00 ± 0.6
	PG38	1.50 ± 0.6	1.50 ± 0.8	1.33 ± 0.3	1.14 ± 1.0	1.14 ± 0.8	1.00 ± 0.5
EC1D + NGR234	PCHA0	1.71 ± 1.0	1.67 ± 0.6	1.33 ± 0.8	1.00 ± 0.7	1.00 ± 0.8	1.00 ± 0.6
	Pf-5	1.50 ± 0.4	1.29 ± 0.7	1.29 ± 0.2	1.17 ± 0.8	1.14 ± 1.0	1.17 ± 0.4
	PG16	1.71 ± 0.6	1.67 ± 0.6	1.33 ± 0.6	1.00 ± 0.5	1.00 ± 0.7	1.00 ± 0.6
	PG19	1.43 ± 0.4	1.50 ± 0.9	1.17 ± 0.7	1.00 ± 0.8	1.00 ± 0.9	1.00 ± 0.4
	PG22	1.67 ± 0.6	1.29 ± 1.0	1.17 ± 0.8	1.14 ± 1.0	1.14 ± 1.0	0.00 ± 0.5
	PG38	1.50 ± 0.4	1.29 ± 0.9	1.75 ± 0.8	1.17 ± 1.0	1.13 ± 0.8	1.00 ± 0.5

Based on the Table 2.6, P-solubilization in *consortia* correlated to individual strain activity. P-solubilization increased substantially along with salt tolerance and was also observed at 5% salt stress as well. Maximum P-solubilization was observed in consortia having Pf-5 (5% NaCl) followed by PG22 and PG38 (4% NaCl). Even though RIC3109 did not grow on the p-deficient media, the consortia exhibit good P-solubilization activity under saline stress. Consortia with RST1 were least affected by salt stress and P-deficiency, however, p-solubilization varied depending on the pseudomonad strain present in the consortium.

A comprehensive analysis for the PGPR trait of individual strains under salt stress is given in Figure 2.9. Salt stress induced increase in the biofilm and IAA production at higher concentrations.

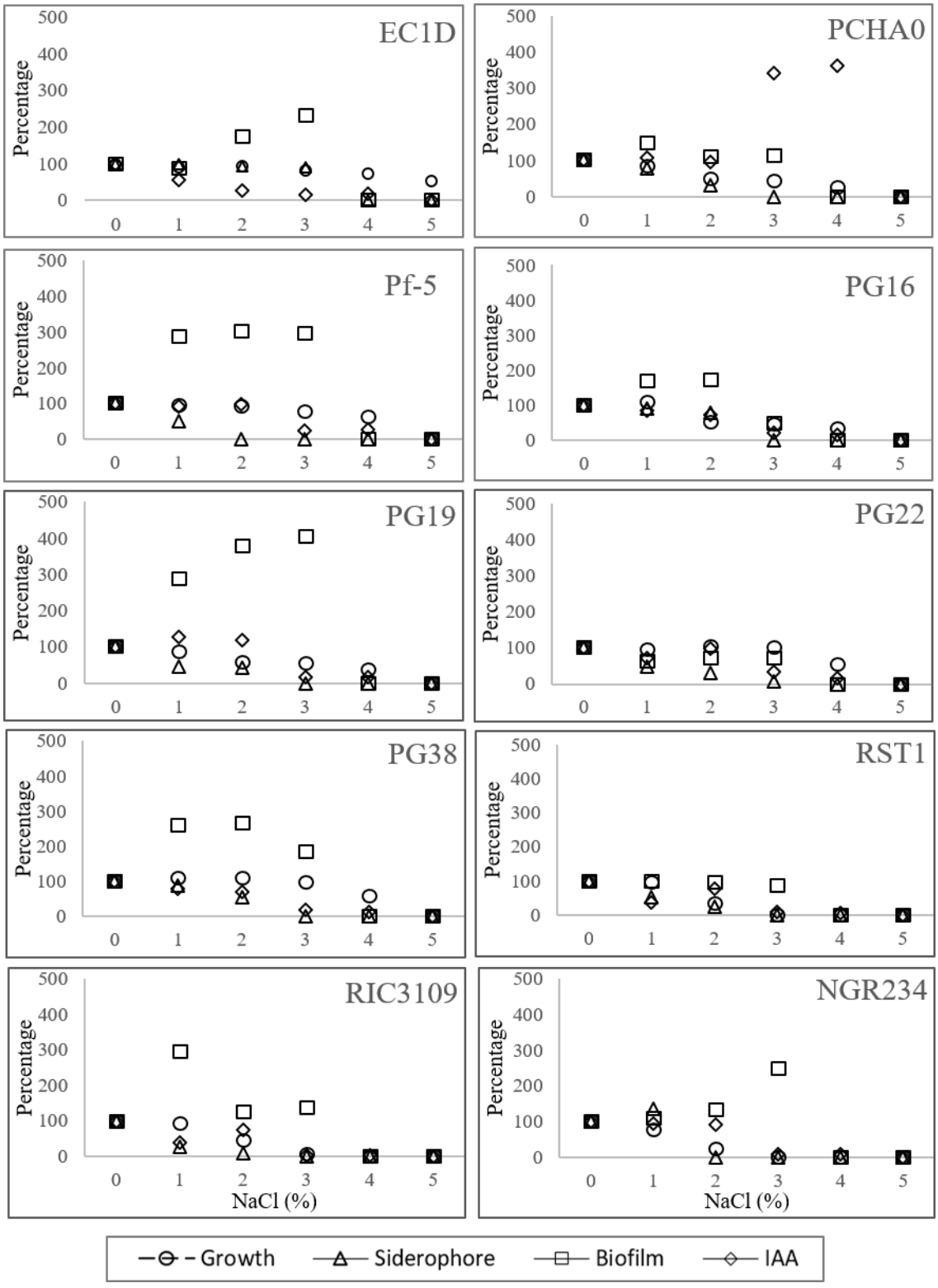

Figure 2.9: PGPR traits under salt stress. The variation in PGPR traits under salt stress were expressed as percent variation from the no salt control sets. The values higher than 100% show an increase in the particular characteristic due to salt stress.

2.3.4.5. HCN production

HCN production is a trait commonly observed in *Pseudomonas* spp. and was absent from other strains in the present study. Among the pseudomonads, only four out of six showed HCN production in variable amounts (Figure 2.10). PCHA0 is known to be a highly positive HCN producer (Chaubey et al., 2015) and was considered as a positive control for the experiment. The strains PG22, Pf-5 and PG38 also showed HCN production with decreasing intensity of gas production in the same order. The stability of gas production under salt stress also varied, PCHA0, Pf-5 and PG22 showed HCN production till 3% NaCl and PG38 till 2% NaCl concentrations. Pf-5 showed the highest HCN production at 2% salinity, while no HCN production was observed at 4% NaCl.

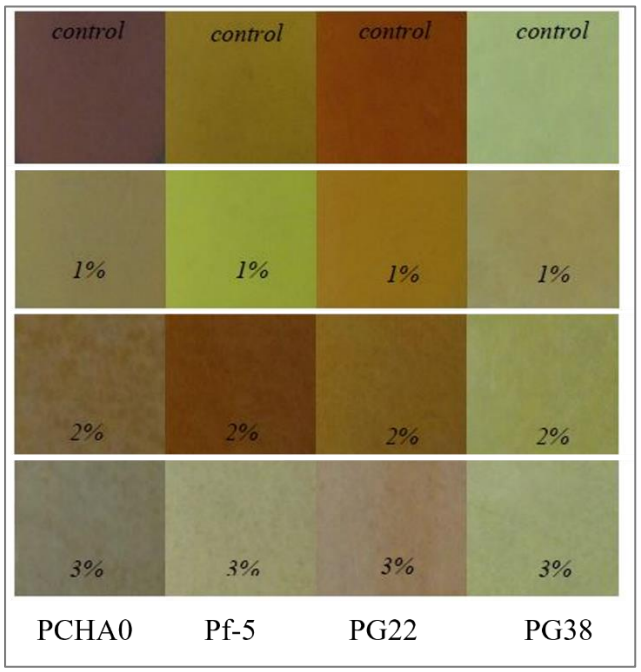

Figure 2.10: HCN production in Pseudomonas trains under salt stress.

Surprisingly, the consortia were unable to produce dark coloration on picric acid-soaked paper discs. It could indicate a synergistic activity among the strains where a low amount of gas production indicated the absence of the need for competition within the consortium.

2.3.4.6. Ammonia production

Ammonia production was observed in EC1D, PCHA0, Pf-5, PG22 and PG38 along with the rhizobia in 0% control. The dark brown precipitate was observed in EC1D and the rhizobia till 2% salinity, PCHA0 showed a decrease in the intensity of colour with increasing salinity. While Pf-5 and PG22 produced orange colouration throughout which indicated the least but

stable ammonia production throughout saline stress, PG38 did not produce ammonia under saline stress. Strains PG16 and PG19 did not produce ammonia at all.

The consortia showed ammonia production, at average intensity (orange colouration), in all the combinations with decreased colour intensity at 1% and 2% salinity, but no ammonia production was observed beyond 2% NaCl concentration. Here, it can be understood that EC1D and rhizobia were the major contributors to the phenotype, whereas discrepancies due to pseudomonads were unidentifiable (Data not shown).

2.3.5. Biocontrol activity

The fungal strains used were found to be not susceptible to salt stress directly in the range used in this study, hence the inhibition using bacterial culture filtrate was entirely due to antagonistic substances produced by the PGPR strains. The pseudomonad isolates showed good inhibition against *F. udum* in no salt condition (Figure 2.11-A). As shown in Figures 2.11 and 2.12, the isolates PG19 and Pf-5 produced high amounts of anti-fungal metabolites in presence of high salt stress. But PG38 did not produce antifungal metabolites against the fungal pathogens, under salt stress.

Antifungal activity in PCHA0 was observed against *R. solani* at 0-2% salinity (Figure 2.12) and F. udum at 1% salinity (Figure 2.11-B). Pf-5 showed antifungal effect at 2% and 3% NaCl concentration against *F. udum* (Figure 2.11-C and D) and *R. solani* at all salt concentrations (Figure 2.12). PG19 and PG22 exhibit antifungal effect against *F. udum* at all salt concentrations. Maximum antifungal activity was observed in PG19 at 4% NaCl and PG22 showed stability at all NaCl concentrations which was equivalent to control (Figure 2.11-E). *Fusarium* inhibition was not observed in PG16 under salt stress.

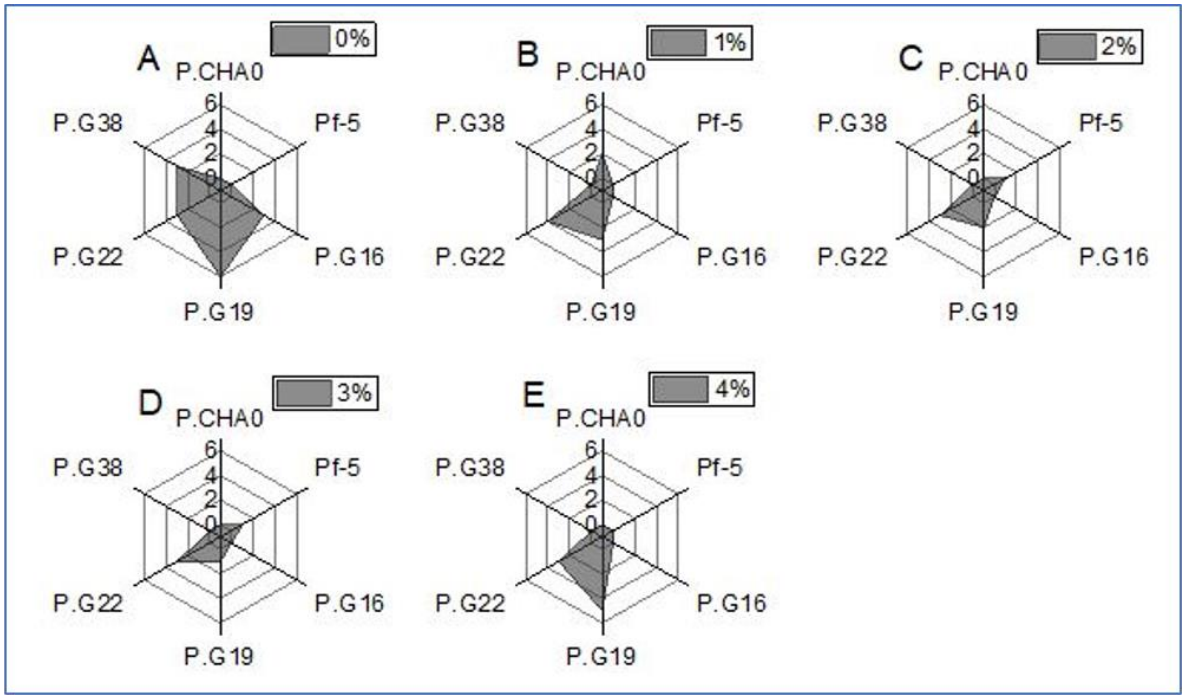

Figure 2.11: Antifungal activity in Pseudomonas spp. against Fusarium udum under increasing salt stress

When compared to *Fusarium, R. solani* was more susceptible to the antifungal metabolites produced by the pseudomonads. PG19 showed antifungal activity at 4% NaCl only. PG22 exhibited antifungal activity at 1% and 2% NaCl (Figure 2.11 - B and C) while Pf-5 was most effective for antifungal activity till 4% NaCl (Figure 2.12).

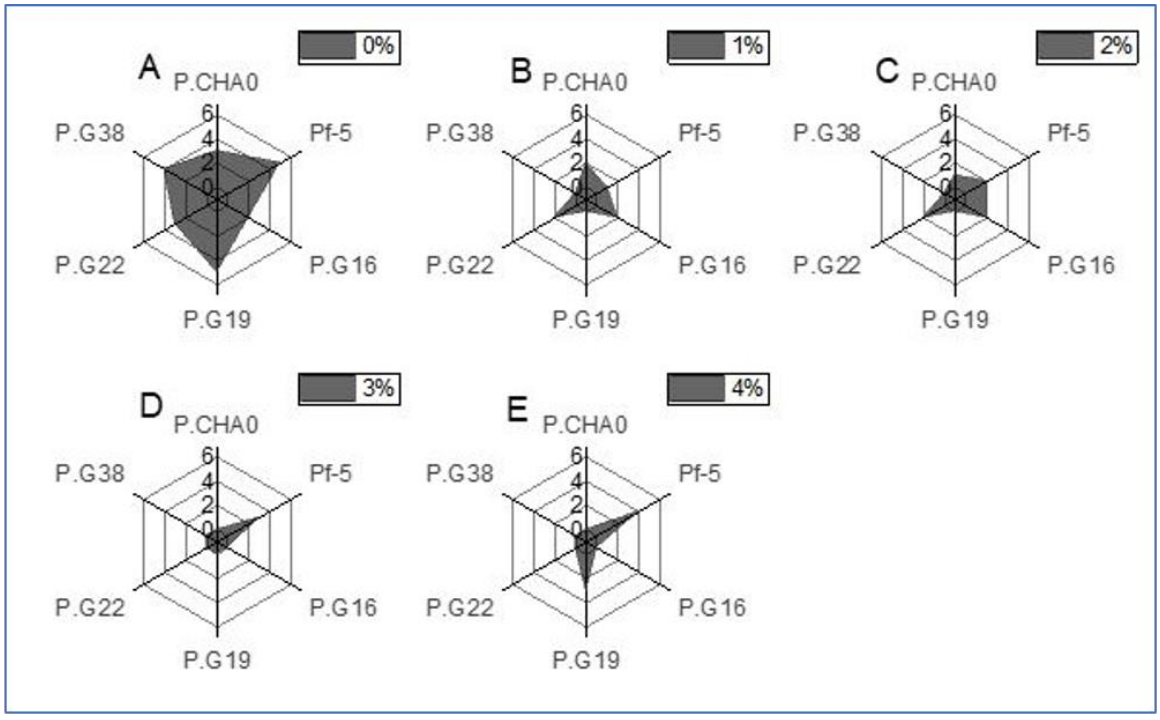

Figure 2.12: Antifungal activity in Pseudomonas spp. against Rhizoctonia solani under increasing salt stress.

For a better understanding of the PGPR behavior of bacterial strains, the PGPR traits of individual strains and their consortia were scored into 4 categories in comparison to the highest value obtained among the strains under study (Figure 2.13).

	Salt (%)	EC1D	PCHAO	Pf-5	PG16	PG19	PG22	PG38	RST1	RIC3109	NGR234
Growth	0	3	3	3	3	3	3	3	3	3	0
	1	3	2	3	3	3	3	3	3	3	0
	2	3	1	3	1	2	3	3	1	1	0
	3	2	1	2	1	1	3	3	0	0	0
	4	2	1	2	1	1	2	2	0	0	0
	5	1	0	0	0	0	0	0	0	0	0
Siderophore	0	3	2	3	2	2	2	3	1	2	2
	1	3	1	1	2	1	1	2	0	0	2
	2	3	0	0	2	1	0	1	0	0	0
	3	2	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
IAA	0	3	1	1	2	1	1	2	3	3	1
	1	2	1	1	1	2	1	1	2	2	2
	2	2	1	1	1	1	1	1	3	3	2
	3	1	2	0	1	0	1	0	1	1	0
	4	1	2	0	0	0	0	0	1	0	0
	5	0	0	0	0	0	0	0	0	0	0
Biofilm	0	2	2	3	3	2	3	3	0	0	1
	1	1	2	3	3	3	3	3	0	2	1
	2	2	2	3	3	3	3	3	0	1	1
	3	3	2	3	2	3	3	3	0	1	2
	4	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
PSE	0	3	2	1	0	0	3	2	0	0	0
	1	2	1	2	0	0	2	0	0	0	0
	2	2	0	1	0	0	1	1	0	0	0
	3	2	0	1	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0
Total		51	29	37	31	29	39	39	18	22	14

Phenotype Score	
0	< 25%
1	≤ 50%
2	≤ 75%
3	> 75%

Figure 2.13: Heat map data representing PGPR activity of the individual strains under salt stress.

EC1D showed maximum salt tolerance among all the strains for growth as well as siderophore production, while the rhizobia were least tolerant towards the stress. IAA production under salt stress was highest in rhizobia, biofilm production was maximum among pseudomonads. P-solubilization efficiency under salt stress was highest in EC1D followed by PG22 and Pf-5.

The tolerance towards salt stress was considerably increased in consortia as compared to individual strains (Figure 2.14). Similar results as individual strains were also observed for

PGPR traits in consortia where the combinations having Pf-5, PG22, PG38 and RST1 had the highest score.

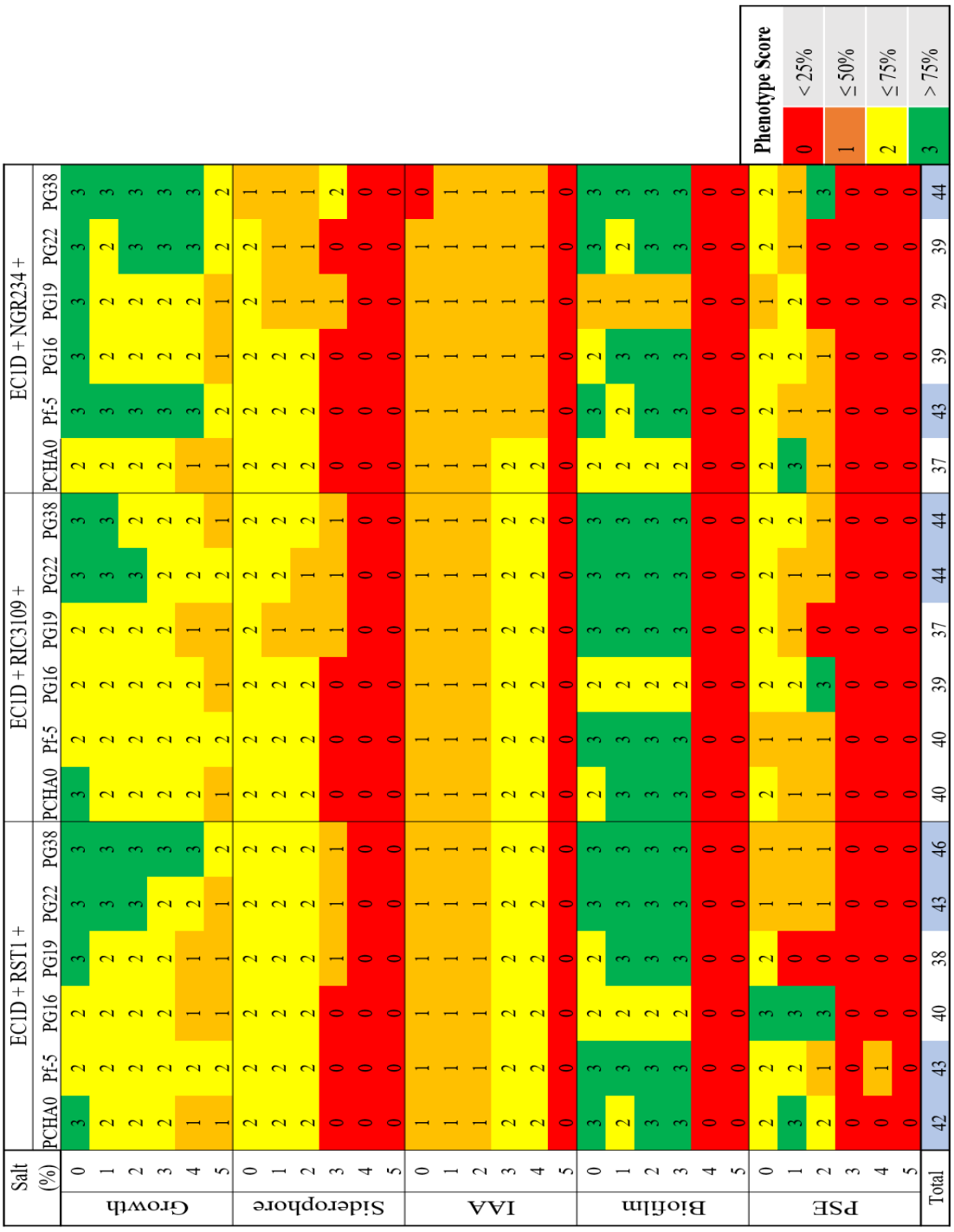

Figure 2.14: Heat map data representing PGPR activity of consortia under salt stress.

2.4. Discussion

While growing in the same environment, bacteria competing for same resources might co-occur, cooperate or exclude each other (Roggenbuck et al., 2015). In case of PGPR consortia, due to these interactions, the PGPR traits may be enhanced or reduced and thus it is important to study the manifestation of PGPR traits in consortia as compared to individual strains. Since the objective of this work was to develop suitable consortia for ameliorating salt stress to plants, it was also imperative to study not only survival but also phenotypic expression of PGPR traits under salt stress.

The level of salt tolerance observed in most PGPR ranges from 100mM – 650mM (0.58% - 3.77%) NaCl, while most crop species are highly susceptible to salt stress not exceeding the range of 60 – 80mM (0.35% - 0.46%) of dissolved ions (Srividhya et al., 2020). All the strains under study by this standard are more resistant than the plants, however among them, highest salt tolerance was observed in EC1D. Thus, this finding, in addition to its previously reported multiple heavy metal resistance, ACC deaminase activity and efficient P-solubilization (Subrahmanyam et al., 2018), suggests its salt tolerating ability. Notably ACC deaminase trait has been associated for salt stress amelioration in plants (Gupta & Pandey, 2019; Nascimento et al., 2016). *Enterobacter* spp. have been reported to alleviate salt stress in many plants (Habib et al., 2016; Kim et al., 2014; Prakash et al., 2017; Sarkar et al., 2018) and have been proven to be *Rhizobium*-helper PGPR (Mirza et al., 2007; Singh et al., 2014).

Pseudomonas spp. were observed to have high salt tolerance after EC1D. Nadeem et al., (2016) reported salt tolerance observed in *P. fluorescence*. *Rhizobium* spp. and NGR234 showed the least salt tolerance among the strains under study. Previous studies conducted on the salt tolerance studies in RST1 (Unni & Rao, 2001) suggested its low tolerance towards salt stress. Although, the salt tolerance levels observed in rhizobia were not up to the mark as other strains, these can also be considered as moderately salt tolerant since these values far exceed the level of salt tolerance in most plant species.

An important aspect of the present study was to assess expression of PGPR traits under salt stressed condition. The microbial compatibility assay revealed no antagonistic interaction among the strains in the current study which ensured competitive stability for consortia development. Pseudomonads have been reported to support other consortial members under stressed conditions stimulating microbial growth and PGPR activity, and display synergistic effect of a consortia over individual strains for plant growth promotion (Korir et al., 2017).

While sometimes, the PGPR traits from one strain, such as IAA production or DAPG production etc., also promotes the consortial stability as a whole (Couillerot et al., 2013). *Pseudomonas* spp. have predominantly shown positive effects as single inocula as well as in mixed cultures for relieving stress in plants such as rice, maize, cotton, soya bean and *Arabidopsis* from salt stress (Chu et al., 2019; Nawaz et al., 2020; Sen & Chandrasekhar, 2014; Vaishnav et al., 2016; Vives et al., 2018). In case of legumes, combinations of rhizobia and *Pseudomonas* have been successfully used as inoculants under saline conditions (Srividhya et al., 2020).

The bacterial strains exhibited varying levels of IAA production. This property could be considered as an important trait in consortium development because the varied time required for IAA production might ensure a continuous hormonal supply for plant growth and development. Kumawat et al., (2019) found a decrease in the IAA production after 3d incubation period along with a synergistic effect for IAA production in the consortia (comprising *Pseudomonas* sp. and *Bradyrhizobium* sp). IAA production by the *Rhizobium* sp. corelated to report given by Karthik et al. (2016), where IAA production reduced under the abiotic stress. In the present study, it was observed that although EC1D and rhizobia strains produced high amount of IAA under salt stress, the consortial IAA production was similar to pseudomonads.

Biofilm formation has been associated with stress tolerance in several studies. Kasim et al. (2016) have reported increased biofilm formation in rhizobia and *Pseudomonas* sp. at 500mM (~3%) NaCl concentration. Primo et al. (2020) have reported increased biofilm formation in *Sinorhizobium meliloti* due to water deficiency stress. Earlier studies conducted for salt stress tolerance on RST1 (Unni & Rao, 2001) have reported deleterious effects of salt stress on EPS production. Similarly Sharaff & Archana (2016) found copper stress which had a negative impact on development of symbiotic association in S*inorhizobium* sp. since it altered the polysaccharide structure which acts as a key signaling molecule for enhancing symbiotic attachment in presence of abiotic stress. Therefore, it is a vital step before the development of a consortium to establish the effects of abiotic stress on this trait to ensure proper bacterial interaction and synergy. Nadeem et al. (2016) have reported biofilm formation in *Pseudomonas fluorescence* up to 5% NaCl concentration, while Fatima et al. (2020) have reported an increment of 263% in the biofilm production in presence of 300mM NaCl in *Alcaligens* sp. The results correlated to ~3-fold increase observed in the *Pseudomonas* sp. in the present study. Zupančič et al., (2018) have discussed the synergistic

effect of multispecies consortia as enhanced biofilm development especially in those having *Pseudomonas* strains.

Most of the strains in this study were able to grow on the P-deficient medium while few developed clear halozone as, but only a selected few (EC1D, Pf-5, PG22 and PG38) were able to solubilize phosphate under the stress as well. Fatima et al. (2020) have also reported P-solubilization till 300mM NaCl in *Alcaligens* sp., while *Pseudomonas* sp. have also been reported to produce clear halo till 5% NaCl (Nadeem et al., 2016). Development of bacterial consortium enabled phosphate solubilization in all the combinations, even though all the member strains did not solubilize phosphate individually. Strain RST1 stimulated maximum phosphate solubilization in all consortia.

Ammonia production helps in nitrogen assimilation and plant growth directly by biomass accumulation (F. Pérez-Montaño et al., 2014). Accumulation of ammonia lowers the soil pH creating an alkaline condition which suppresses the growth in certain fungi and inhibits spore germination as well (Swamy et al., 2016). Most of the strains under study demonstrated ammonia production under salt stress individually as well in consortia under salinity stress up to 2% salinity.

The standard strain *Pseudomonas* sp. CHA0 has been reported for the biocontrol activity against several root pathogenic fungi including *Fusarium udum, Rhizoctonia solani, Rhizoctonia bataticola,* and several others, the key factor being production of the antibiotic 2, 4-DAPG (Chaubey et al., 2015; Schnider-Keel et al., 2000; Voisard et al., 2007) along with several secondary metabolites such as HCN production (Rijavec & Lapanje, 2016). *Pseudomonas fluorescence* Pf-5 is a rhizospheric bacteria also known for disease suppression abilities via production of several antimicrobial compounds including 2, 4-DAPG, pyoluteorin, pyoverdine, HCN, etc for biocontrol activity (Schnider-Keel et al., 2000). Bacterial cyanogenesis is reported in most of the fluorescent pseudomonads, in variable amounts depending on the environmental factors (Schippers et al., 1990). HCN production occurs under microaerophilic conditions, while the fluorescent pseudomonads themselves are resistant to the cyanide up to a certain limit (Blumer & Haas, 2000; Pessi & Haas, 2000). Pathogenic effects of HCN in *Pseudomonas* spp. for biocontrol effect has also been associated with chelation of metal ions, and increasing the availability of phosphorus which is beneficial for the rhizobacteria and their plant hosts (Rijavec & Lapanje, 2016). The present study illustrates salt tolerance in pseudomonads for HCN production trait, especially in the

standard strains PCHA0 and Pf-5 along with 2 other pseudomonad isolates. Interestingly, HCN production in consortia could not be detected. In the present study, high salt tolerance was observed among the pseudomonads and their PGPR traits including HCN, siderophore and ammonia production. Production of antifungal molecules was also observed in the pseudomonad strains under salt stress, which was higher in some of the strains. This phenotype has been reported by Egamberdieva et al. (2017), where *B. subtilis* showed biocontrol of chick pea root rot in saline soil which was attributed towards combined effect of antagonistic traits.

Siderophore production by the PGPR ensures its availability to plants while its competitive occlusion of iron helps in bio control activity against plant pathogens Salt affected soils (SAS), with high Na^+ concentration makes it even more difficult for the plants to absorb iron (Abbas et al., 2015; Rout & Sahoo, 2015). With the exception of EC1D, siderophore production in individual strains decreased with increasing salt stress and there was no siderophore production among these at 3% salinity. Strain specific differences in sensitivity of bacteria to secrete siderophores have been observed (Fatima et al. 2020; Nadeem et al. 2016). While the consortia were least affected up till 2% salinity and siderophore production was observed at 3% salinity as well which demonstrated synergy among the bacterial strains.

Siderophores mediate competitive environment in the rhizosphere which also influences microbial interactions (Niehus et al., 2017). While absence of appropriate siderophore receptors can induce iron-starvation (Kramer et al., 2020). The ability to cross-utilize siderophores from heterologous sources help overcome the nutrient stress and was attributed towards presence of multiple siderophore receptors providing a broad host-range specificity (Khan et al., 2006). Zupančič et al., (2018), have reported that highest number of positive co-relations were observed in consortia having *Pseudomonas* strain, suggesting high potential in them to co-exist with bacteria from other genera. These results were similar to the present study, where the pseudomonad strain was found to dictate the siderophore production in consortia under salt stress.

To summarize, most of the PGPR traits in individual strains were completely inhibited at salt concentrations where bacterial growth was not yet inhibited. Among all the strains under study, EC1D was found to exhibit high tolerance and phenotypic demonstration of the PGPR traits (P-solubilization, siderophore and IAA production) under salt stress. Pseudomonads Pf-5, PG22 and PG38, demonstrated maximum tolerance for the PGPR traits such as biofilm, P-

solubilization including biocontrol activity such as HCN production, antifungal activity. Rhizobial strains RST1 and RIC3109 showed a few similar PGPR characteristics (IAA production, ammonia) including growth conditions. The consortia demonstrated enhanced salt tolerance towards exhibition of PGPR traits under salt stress. Biofilm and IAA production were found to be enhanced by several folds at higher salt concentrations especially in the consortia having Pf-5, PG22 and PG38 strains.

Based on the results obtained for PGPR traits of individual strains under salt stress, these six PGPR ie., EC1D, Pf-5, PG22, PG38, RST1, RIC3109 and NGR234 were selected for further experiments related to bacterial interaction in consortium and their survival under stress conditions.