Chapter 3

Functioning and compatibility of the PGPR consortia in presence of multiple abiotic stresses using PCR based approach

3.1. Introduction

Functionality and effectiveness of a consortium is dependent on multiple factors including (1) bacterial interactions which may be synergistic or antagonistic, (2) net contribution of each individual member – which may or may not be affected by the presence of other species in a consortium and (3) population size of each member after a particular time period determined by the ability to survive the ecological competition, which in turn determines the net contribution relative to their monocultures (Sanchez-Gorostiaga et al., 2019).

Saline soils limit the bioavailability of nutrient elements because of the modified ionic balance. Bioavailability of soluble phosphates is restricted due to its fixation as insoluble forms, in acidic soils it gets converted to iron and aluminium phosphates while in alkaline soils it is mainly converted calcium phosphates (Alaylar et al., 2020). Application of phosphate solubilizing bacteria (PSB) has become a most promising option for decreasing input of chemical fertilizers lower production cost while at the same time enhancing phosphate bioavailability and efficient utilization by the plants without exacerbating the soil salinity (Backer et al., 2018; Etesami & Beattie, 2017). Additionally, production of HCN, which has been considered as only as a plant protective mechanism, has also been associated to enhancing phosphate availability to the plants. Rijavec & Lapanje (2016) have indirectly shown that the cyanide plays an important role in dissolution of phosphorus, while the plant biomass content was also significantly higher in presence of HCN positive PGPR strains. Siderophore production associated with creating a competitive atmosphere by nutrient deprivation has also been linked with phosphate solubilizing activity and could be increased via metal chelation (Jaiswal et al., 2021). Nandre et al. (2021) have shown 99% recovery of phosphate using *Pseudomonas* strains as the source of siderophores based on struvite biomineralization.

Molecular techniques have been increasingly applied for the quantification of microbial communities which include denatured gradient gel electrophoresis or DGGE (Subrahmanyam et al., 2011), flow cytometry (Müller & Nebe-Von-Caron, 2010), proteomics and metagenomics (Jia et al., 2016). PCR and qPCR techniques provide a high dynamic range for cell quantification and multiplexing for several targets can also be performed in a single reaction, for targeting specific regions or genes and can also be applied based on phenotypic and functional characteristics (Kralik & Ricchi, 2017).

Amplicon sequencing based on the small subunit rRNA (16s in bacteria) analysis due its ubiquity, allows the scope of applying a broad range of various techniques and collection of large number of samples simultaneously for population dynamics studies (Sinclair et al., 2015). In this Chapter, 16s rRNA partial gene sequence analysis by the qPCR method was carried out to assess the consortial members and understand the inter-relationship among individual strains and their perseverance under multiple (nutrition and/or salinity) stressed conditions. PGPR strains characterized in the previous Chapter were used for the further experiments.

3.2. Materials and Methods

3.2.1. PGPR consortia

The consortia were prepared by adding same concentration of each PGPR strain as in the individual experiments. The individual strain experiments were carried out as reference against performance of each strains in different combinations. The consortia using selective strains mentioned in Table 2.1, Chapter 2 were designated as given in the Figure 3.1.

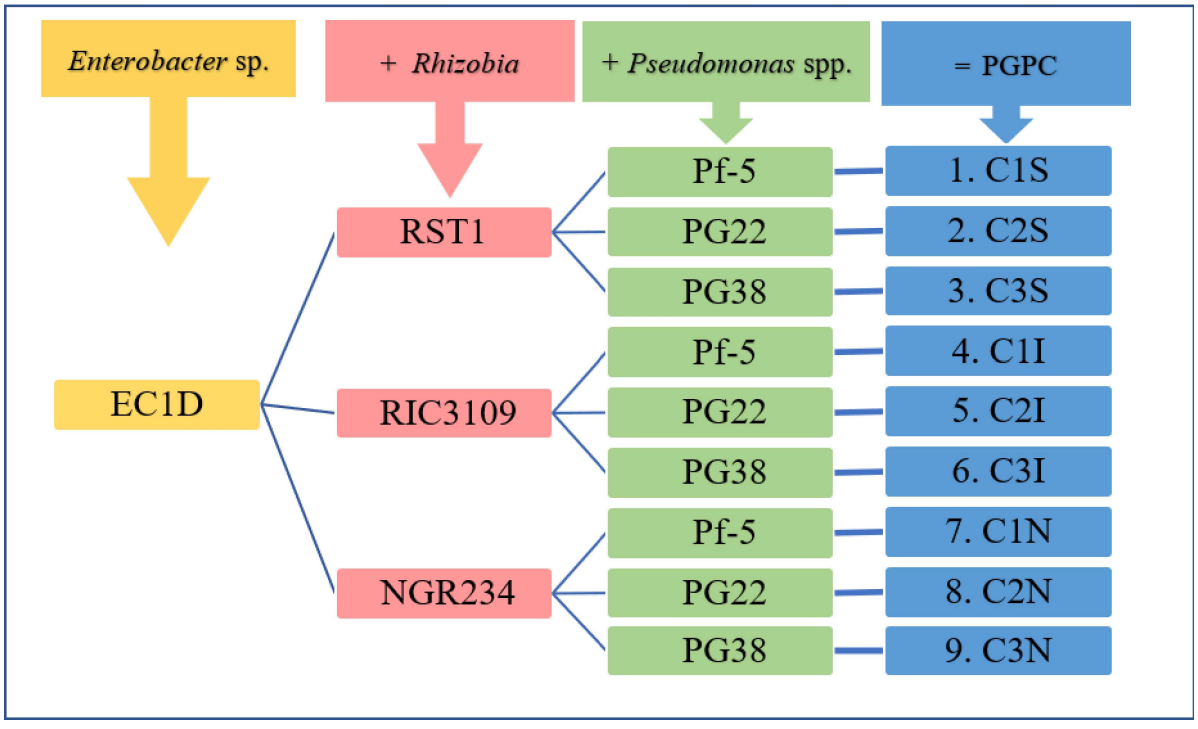

Figure 3.1: Consortia of PGPR strains used in this chapter. Enterobacter sp. EC1D was common to all three membered plant growth promoting consortia (PGPC), which contained in addition one of the three rhizobial strains and one of the three Pseudomonas strains giving rise to 9 different combinations.

3.2.2. Bacterial growth in phosphate starved conditions and salt stress

The consortia were subjected to growth experiments in salt stressed conditions using Luria Bertani (LB) broth and in Tris-Rock phosphate (TRP) medium [M9 minimal medium supplemented with washed Senegal rock phosphate (RP) as the sole P source, buffered by 100 mM Tris–Cl pH 8.0, NH_4Cl and 100 mM glucose] (Buch et al., 2008) under salt as well as P-starvation stress. The salt stress was applied at concentrations 0 – 5% NaCl (w/v). The bacterial strains were grown individually and given the same treatment as consortia for comparative studies. Individual strains grown in the absence of NaCl were used as reference for the quantitative analysis. The overall bacterial growth was monitored by OD measurements. P-solubilization was monitored in TRP medium by release of P in supernatant and pH of the supernatant was measured as an indicator of organic acid secretion. The inoculum (Section 2.2.3) was aseptically added to the medium flasks and kept for incubation in shaking condition 120rpm at 30°C for indicated time.

3.2.3. Quantification of the released inorganic phosphate

A stock concentration of 0.33mM KH_2PO_4 was used as the standard for inorganic phosphate. The concentration of the soluble phosphate in the supernatant was estimated every 12h using the Ames method (Ames, 1966).For quantitation, 0.70 ml of the reagent [10% ascorbic acid was mixed with 0.42% ammonium molybdate.$4H_2O$ (1:6 ratio) in 1 N H_2SO_4 and kept in ice] mixed with 0.30 ml of cell-free supernatant (0.30 ml of double distilled water was used as a blank) and incubated for 20 min at 45°C in the water bath, change in colour was measured at OD_{820nm} using the UV spectrophotometer (Shimadzu 1800).

3.2.4. Designing of strain specific primers based on 16S rRNA gene sequence

The growth of individual members of the consortia was monitored by PCR using specific primers for each strain. Genus specific primers were designed for each of the 3 groups of bacteria (*Enterobacter* sp., *Pseudomonas* sp. and *Rhizobium* sp.) based on the 16S rRNA partial gene sequence (Section 2.2.2.) using the Primer3 software (an online tool for primer designing). The primers were then checked for cross-specificity using NCBI-Primer BLAST and the PCR method to rule out the primers forming multiple bands or non-specific amplification.

The primers were designed in accordance to obtaining a final amplification product of size ≥300bp. The size of each amplified product was small enough to be useful for complex studies such as qPCR. The forward (f) and reverse (r) primer sets designed for the *Enterobacter* sp. included E326f and 520r, for *Rhizobium* spp. were R43f and R254r and

those for the *Pseudomonas* spp. was P213f, paired with DGGE primer 517r (Ikenaga & Sakai, 2014). The NGR234 primers included NGRf and NGRr. The primer details are given in the Table 3.1.

Table 3.1: PCR primers used in this study.

Target	Primer	Sequence (5' to 3')	Amplicon size (bp)	Tm (°C) (XX)	Reference
16s rRNA partial gene amplification					
16s rRNA gene	27F	AGAGTTTGATCCTGGCTCCAG	1080	58.0	Subrahmanyam et al., 2018
	1107R	GCTCGTTGCGGGACTTAACC			
Quantification by PCR method					
Enterobacter sp.	326 F	AAACGATGTCGACTTGGAGGTT	194	63.3	This Study
	520 R	GGACACCTATACGCATCTCTGCT			
Rhizobium spp.	43 F	CCGTGATAGCTAGTGGTGGG	211	63.3	
	254 R	GGGCTTCTTCTCCGACTACC			
Pseudomonas spp.	213F	ATCTTCGGACCTCACGC	~300	58.2	
	517 R (DGGE)	ATTACCGCGGCTGG			
E. fredii NGR234	NGR F	GTGTGCCAGCTTATTGGCG	~300	58.2	
	NGR R	TCAAAGACATATGATGCCGTTGCTC			

3.2.5. DNA extraction from individual strains and mixed consortia

DNA extraction was performed with 2ml of the bacterial cultures collected in centrifuge tubes at 24h and 48h interval respectively, by the modified C-TAB method (Sambrook & Russell, 2001). The bacterial cell cultures were resuspended in 330µl of TES. To this 5µl of lysozyme (20mgL^{-1}) was added and the mixture was kept in water bath at 60°C for cell lysis till 45 min. After cooling down to RT, 30µl of 10% SDS (w/v) and 6µl Proteinase-K (10mgL^{-1}) was added and mixed by inverting the centrifuge tubes, kept in the water bath at 60°C for

30 min for digesting. To this, 150µl 5M NaCl along with 80µl 10% (w/v) cetyltrimethylammonium bromide (C-TAB) was added and incubated for 15min at 60°C. 400µl of Phenol-Chloroform-Isoamyl alcohol (25:24:1 ratio) was added, and centrifuged to separate out the DNA from debris. The upper clear phase was treated with 300µl of Chloroform-Isoamyl alcohol mixture (24:1 ratio) and centrifuged at 14,000 rpm for 20min. The upper clear phase was mixed with $1/10^{th}$ volume of 3M sodium acetate and double the volume of chilled absolute ethanol. The vials were then shifted to -20°C for overnight storage. The DNA pallet was centrifuged 10k rpm, the DNA pallet was separated by discarding the supernatant. The pellet was washed using 300µl of 70% ethanol gently, centrifuged air-dried using Savant DNA 120 SpeedVac (Thermo Scientific, India) at medium heat.

The precipitate was resuspended in 50µl sterile Ddw water with 1µl RNase treatment. An aliquot of 15µl from each sample was saved at 4°C for analysis while the rest of were stored at -20°C until further requirement. The samples were quantified (ng/µl) by the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, India), run on gel electrophoresis in 0.8% agarose using 1X TAE buffer. Stock solutions were then diluted (20µl volume) to a final concentration of 10ng/µl and stored at 4°C for the semi-quantitative analysis experiments, the remaining stocks were maintained at -20°C.

3.2.6. PCR amplification and semi-quantitative estimation

PCR amplification from the conserved bacterial 16s rRNA region, was conducted using the primers mentioned above. PCR reactions were carried out in 10µl volume, using the genomic DNA (gDNA) from sample aliquots as the DNA template. The reaction master mix was prepared by mixing 30µl of 10X Taq DNA polymerase buffer (with $MgCl_2$), 6µl of 10mM dNTP stock, 25µl each of the forward and reverse primers (10µM stock) and 6µl of Taq DNA polymerase (5U), making up the final volume to 185µl using sterile Ddw. The master mix was then distributed into 30 vials along with 1ng respective templates (10µg/µl diluted stock).

Amplification was carried out in a thermal cycler (Applied Biosystems) with an initial denaturation at 94°C for 3min, followed by 35 cycles, each consisting of denaturation at 94°C for 45s, annealing at temperature specific for the primer set for 30s, elongation at 72°C for 45s, followed by a final elongation at 72°C for 10min. Amplicons were detected by electrophoresis method on 1.8% (w/v) agarose gels in TAE buffer using ethidium bromide

(EtBr). The amplified products of each bacterial strain (in consortium), were compared with individually grown bacterial strains grown under similar conditions as reference. The bands were semi -quantified based on the relative difference in intensity using the Image Lab software (version 6.1).

3.2.7. Bacterial growth under salt stress - Quantitative PCR assay

3.2.7.1. PGPR growth under salt stress

The bacterial strains were grown in consortia in 30ml LB broth. Sample aliquots were collected at 3h intervals up to 24h of growth period. At an interval of 12h, aliquots were also collected for gDNA extraction and bacterial cell count estimation. The individual strains were subjected to the same treatment as control sets.

3.2.7.2. Standard Preparation

The DNA templates from pure cultures (EC1D, Pf-5, RST1 and NGR234) were amplified by the quantitative PCR (qPCR) method using the respective primers. The PCR amplicons were purified by glass wool method and used for the transformation, the protocol for transformation was adapted from Sambrook & Russel (2001). The *E. coli* DH5α cells were used for the comp cell preparation and the amplified PCR products were ligated onto pJET1.2/blunt cloning vector as per manufacturer's instructions (Thermo scientific, India). The insert concentration and sample volume were determined by the following formulae (Equation 2).

$$1M\ Insert\ (ng) = \frac{Kb\ of\ Insert}{Kb\ of\ vector} \times ng\ of\ vector\ (1M) \qquad \textit{(Eq. 2)}$$

where, kb of insert = 0.3 kb for *Pseudomonas* spp. and NGR234,
0.2 kb for *Rhizobium* spp. & *Enterobacter* sp.
kb of vector = 2.886 kb
ng of vector = 55ng/ µl

1M insert volume = 1M concentration (ng) / product concentration (ng/µl). *(Eq. 3)*

The molar ratio of insert to vector was adjusted to 3:1 (Equation 3) for an ideal ligation reaction. For each separate reaction, the ligation reaction was prepared by adding 2µl Ligation buffer (5X), 6µl Insert (3M), 1µl each Vector (1M) and T4 DNA Ligase (1000 U). The vector & insert were first pooled and warmed at 45°C for 5 min to melt any cohesive termini that had re-annealed during storage. It was then chilled on ice for 2 min and ligated

with (1000 U) high-concentration T4 DNA ligase (Thermo-Fisher) in 1X T4 DNA ligase buffer. The 10µl reaction mixtures were incubated in Ligation bath at 22°C for 2 hours followed by inactivation at 65°C for 10 min, & then set at 4°C until transformation. For negative control, control DNA 1 (2886 bp; 0.1µg/µl; circular DNA without insert) was used, and for positive control – control DNA 2 (2886 bp; 0.1µg/µl; circular vector + control PCR insert). These were diluted separately (0.5µl / 9.5µl d/w) and set on ice till transformation.

The competent cells (2ml volume) were distributed (200µl each) into 10 pre-chilled ligation vials (with ligation mixture) and kept on ice (4˚C) for 30min. It was transferred to 42˚C / 90sec (water bath) for heat shock and chilled (2 -3 min). To each vial, 800µl sterile LB broth was added aseptically and placed on a shaker at 37˚C for 45min. The bacteria were screened by Blue-white colony screening method to isolate truly transformed ones. LB agar plates amended with 50µg/ml Ampicillin layered over by 40µl each of X-gal (20mg/ml) and IPTG (24mg/ml) respectively. 100µl of comp cells prepared above were spread plated on these agar plates and incubated at 37˚C overnight. The plates were shifted to 4˚C, and isolated colony from each set was picked and re-inoculated into fresh LB-Amp tubes for plasmid isolation.

The recombinant *E. coli* cells, transformed with plasmid vector carrying amplified products from each primer set respectively, were subjected to plasmid extraction by the alkaline lysis method (Sambrook & Russel, 2001). Reagent preparation for the same has been mentioned in Appendix I. The recombinant plasmid DNA was quantified in NanoSpec for copy number calculation based on Equation 4.

$$\text{DNA Copy No.} = \frac{\text{DNA concentration (X) (ng/µl)} \times 6.0221 \times 10^{23}\ \text{(copy/mole)}}{\text{Insert length (Y) (bp)} \times 660\ \text{(g/mol)} \times 1 \times 10^{9}\ \text{(ng/g)}}$$

(Eq. 4)

where, X is the DNA concentration of the samples calculated from NanoSpec readings, and

Y was calculated as vector + amplicon size

Based on the copy number value, the samples were serially diluted in concentrations ranging from 10^2 to 10^8. These serial dilutions were used as template for qPCR analysis in triplicate sets to prepare Ct-threshold values standard graph using StepOne Real-Time PCR system (Applied Biosystems, 7300). TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara, India) was used for setting up the PCR reactions and the reactions were carried out using MicroAmp

48well plates and optical adhesive films (ThermoFisher Scientific, India). Each 480µl master mix reaction contained 2X TB Green premix (240µl), Rox dye (5µl), forward and reverse primers (15µl each respectively) and the volume made up using Ddw. The reaction was set up on ice in aseptic conditions, master mix was mixed thoroughly by vortexing and distributed (9µl) to the MicroAmp 48-well plate. 1µl template (10ng/µl concentration) was added respectively to each predefined well and the plates sealed with adhesive films. Centrifugation was carried out, to remove air bubbles, at 2000 rpm / 1min and the plates were shifted into the thermal cycler. The PCR reaction was as given in the Figure 3.2. Initial denaturation at 95˚C (30sec) followed by the PCR reaction of 40 cycles, consisting of denaturation at 95˚C (20sec), annealing at XX˚C (30sec; XX indicates annealing temperature was set based on primers used), elongation at 72˚C (30sec) and lastly the melt curve cycle with denaturation (95˚C / 15sec), renaturation (80˚C / 1min) and denaturation (95˚C / 15sec).

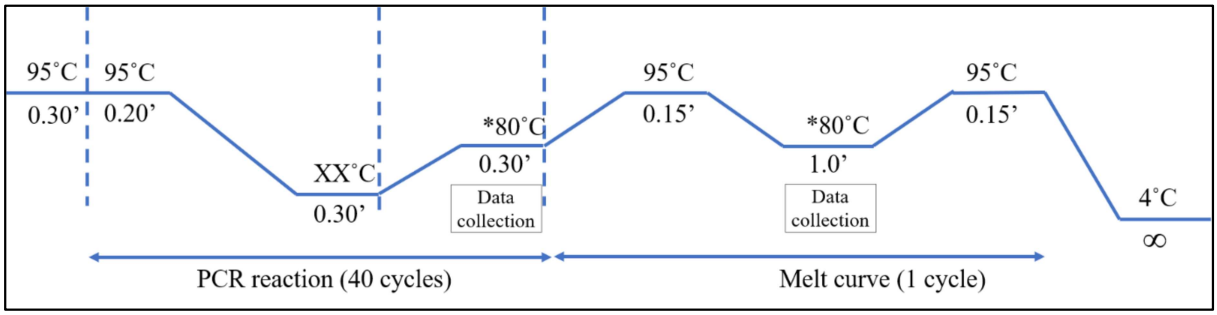

Figure 3.2: Real Time PCR reaction cycle for amplification of 16S rRNA from PGPR strains in consortia.

* - indicates stage of data collection for fluorescent readings.

The Ct-value obtained for each concentration was used for the preparation of standard graphs of each primer set (Appendix II).

3.2.7.2. Q-PCR Analysis for bacterial growth establishment

The sample aliquots collected from the growth experiments were subjected to gDNA extraction and sample preparation (Section 3.2.5.). The samples were quantified using the NanoSpec and diluted to a concentration of 10ng/µl for q-PCR assay. As mentioned in the previous Section, the qPCR reaction for each strain was carried out using respective primer sets and based on the standard graph the Ct values obtained were used for copy number estimation for each strain in the consortia at different salt concentrations and time periods.

3.3. Results

3.3.1. Phosphate solubilization by individual PGPR strains under salt stress

Among all the strains, EC1D showed maximum P-solubilization under salt stress at upto 4% salinity (Table 3.2). Strains RST1 and RIC3109 were unable to solubilize phosphate while NGR234 solubilized the rock phosphate only under no salt condition. P-solubilization by PG38 was inhibited beyond 2% salinity, Pf-5 and PG22 solubilized phosphate till 3% NaCl. PG22 and PG38 had the highest amount of phosphate solubilization (48h growth period).

Table 3.2: P-solubilization by the individual PGPR strains under salt stress. The values in red indicate highest values under each salt percentage. The letters in superscript indicate significant differences within each salt concentration and the Asterisk (*) indicates significant differences among highest values in each strain at different salt concentrations. The significance was calculated using ANOVA single factor analysis (P≤0.05).

PGPR Strains	Maximum P-release (mmol/L, Time in hours)				
	0%	1%	2%	3%	4%
EC1D	1.217 (72h)^{a*}	0.918 (36h)^{a*}	0.895 (48h)*	0.679 (36h)**	0.679 (60h)**
Pf-5	0.818 (60h)^{b*}	1.002 (84h)^{b*}	0.838 (60h)*	0.380 (72h)	0.156 (48h)
PG22	1.462 (48h)^{c*}	0.864 (60h)^{c**}	0.818 (84h)**	0.377 (60h)	--
PG38	1.465 (48h)^{c*}	0.704 (60h)^{d**}	0.617 (84h)**	--	--
RST1	--	--	--	--	--
RIC3109	--	--	--	--	--
NGR234	0.570 (72h)*	0.307 (84h)**	0.119 (12h)	0.137 (24h)	0.133 (36h)

Figure 3.3 shows time dependent P release and the pH of the culture supernatants. In spite of fluctuations seen, a gradual increase in P release was observed up to 60h time period. EC1D and Pf-5 showed maximum pH drop within 12h of growth at all salt concentrations.

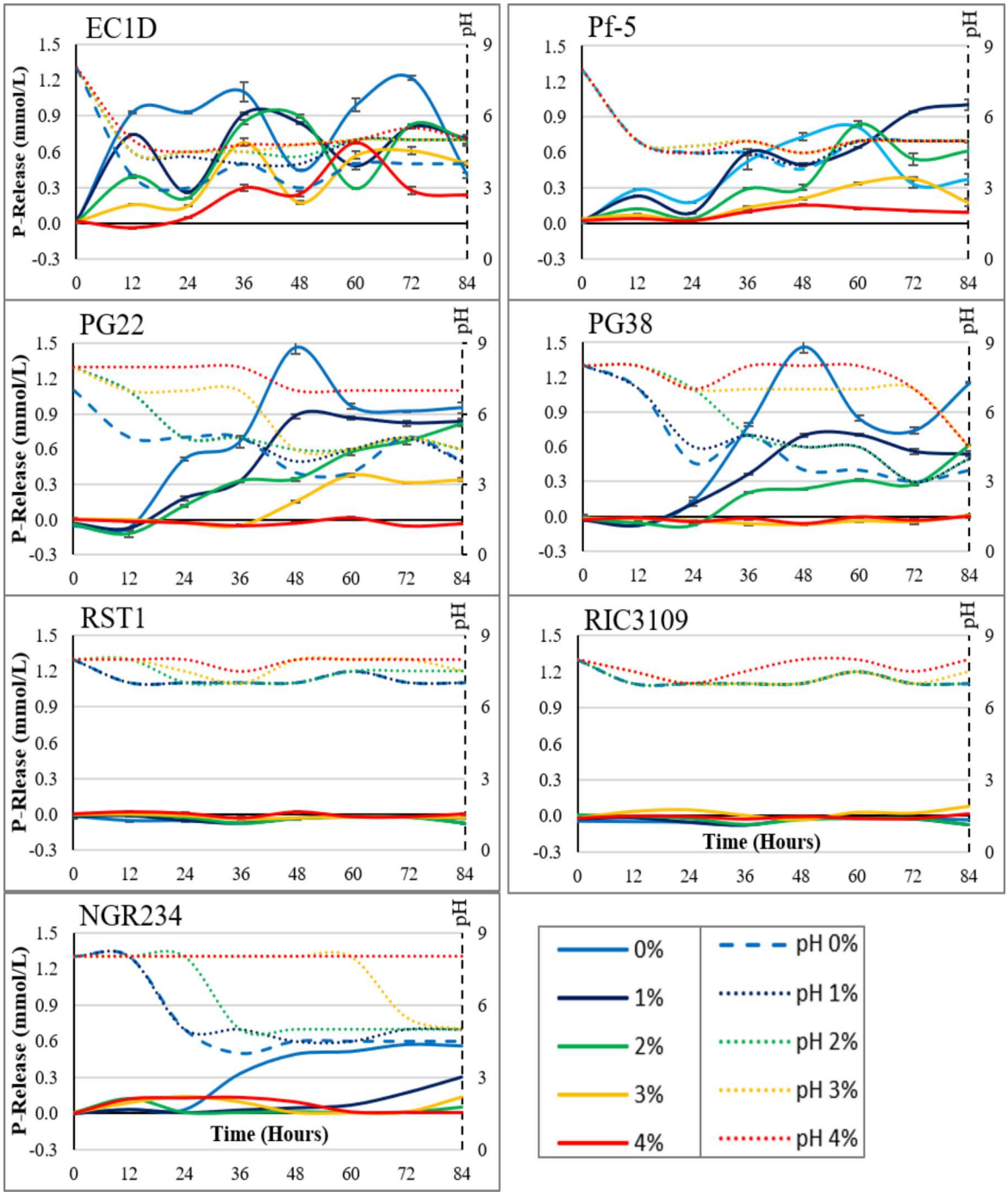

Figure 3.3: P-release and pH of the culture supernatants in individual PGPR strains under salt stress. pH change is indicated in dotted lines.

The P-solubilization was corresponding to pH drop of the medium. It was absent in RST1 and RIC3109, similarly there was no pH drop in the broth medium. NGR234 solubilized phosphate only in no salt stress medium where a decrease in broth pH was observed till 2% salinity and delayed effect at 3% salinity was observed as well.

3.3.1.1. Phosphate solubilization by PGPR consortia under salt stress

The overall P-release was enhanced by 2-folds among the consortia, while P-release at 4% salt stress was found to be low in consortia as well (Table 3.3). Although Pf-5 showed P-solubilization at 4% salinity, it was absent in consortia except with NGR234. It was observed that PG22 and PG38 showed high P-solubilization as individual strains and similarly, all the consortia having these strains have shown higher P-solubilization in no salt control and salt stress.

Table 3.3: P-solubilization by PGPR consortia under salt stress. The values in red indicate highest values obtained under each salt percentage. The letters in superscript indicate significant differences within the different consortia at each salt concentration and the Asterisk (*) indicates significant differences among the highest values in each consortium at different salt concentrations. The significance was calculated based on ANOVA single factor analysis (P≤0.05).

PGPR Consortia	Maximum P-release (mmol/L, Time in hours)				
	0%	1%	2%	3%	4%
C1S	1.621 (84h)^{a*}	1.714 (48h)^{a*}	1.516 (48h)^{a*}	1.436 (72h)^{a*}	--
C2S	2.422 (84h)^{b*}	2.521 (84h)^{b*}	2.517 (84h)^{b*}	1.759 (84)^{ab**}	0.115 (84h)^{a}
C3S	2.416 (60h)^{b*}	2.614 (60h)^{b**}	2.213 (60h)^{c*}	1.757 (84h)^{a}	0.216 (84h)^{b}
C1I	3.286 (84h)^{c*}	1.642 (48h)^{a**}	1.359 (48h)^{a}	1.263 (72h)^{b}	--
C2I	2.757 (84h)^{b*}	1.851 (72h)^{a**}	2.056 (72h)^{c**}	1.730 (84h)^{a**}	--
C3I	2.538 (60h)^{b*}	2.605 (60h)^{b*}	2.269 (60h)^{c**}	1.607 (60h)^{ab}	0.201 (84h)^{b}
C1N	1.095 (84h)^{d*}	0.892 (84h)^{c**}	0.668 (84h)^{d}	0.504 (84h)^{c}	0.176 (84h)^{c}
C2N	1.287 (84h)^{e*}	1.013 (84h)^{d**}	0.764 (84h)^{e}	0.577 (84h)^{d}	0.216 (84h)^{b}
C3N	1.377 (84h)^{f*}	1.073 (84h)^{e**}	0.796 (84h)^{f}	0.169 (84h)^{d}	0.101 (12h)a

The consortia having NGR234 as the rhizobia component also showed similar pattern for phosphate solubilization at all salt concentrations (Figure 3.4). With the exception of C2N and C3N, there was no pH drop at 4% salinity, while decrease in the pH was corresponding to P-release in the consortia as well and at all the salt concentrations.

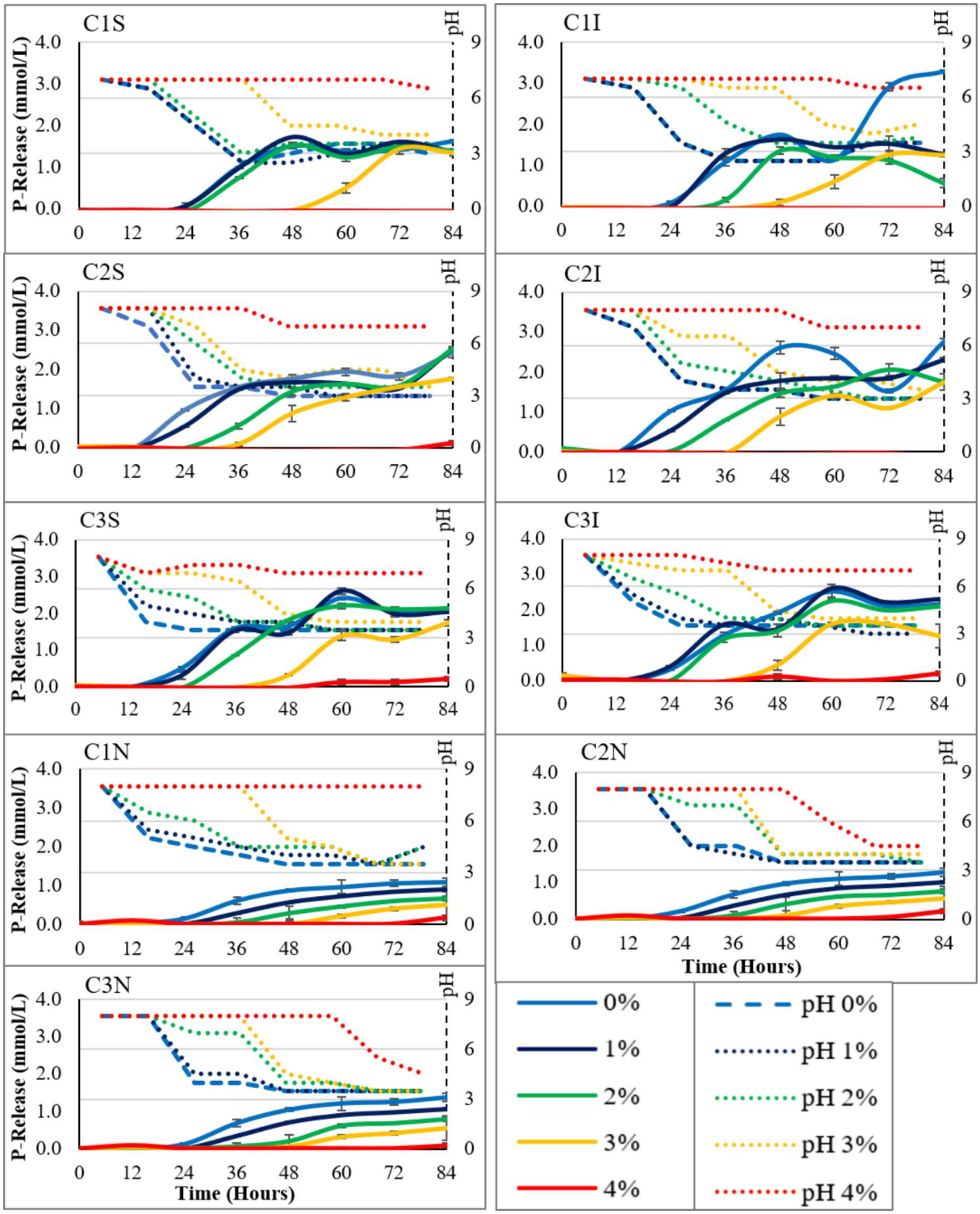

Figure 3.4: P-release and pH from the culture supernatants in consortia under salt stress. pH values are indicated as dotted lines.

3.3.2. Bacterial growth under P-deficiency and salt stress

Rhizobium sp. showed highest optical density in the dual stress condition, while decrease in growth was also observed due to salt stress (Figure 3.5). At 3% salinity, growth activity was observed only in PG22 and NGR234, which was totally absent at 4% NaCl. The bacteria

attained stationary phase within 24h to 48h of growth, therefore, the sample aliquots were also collected at these time periods.

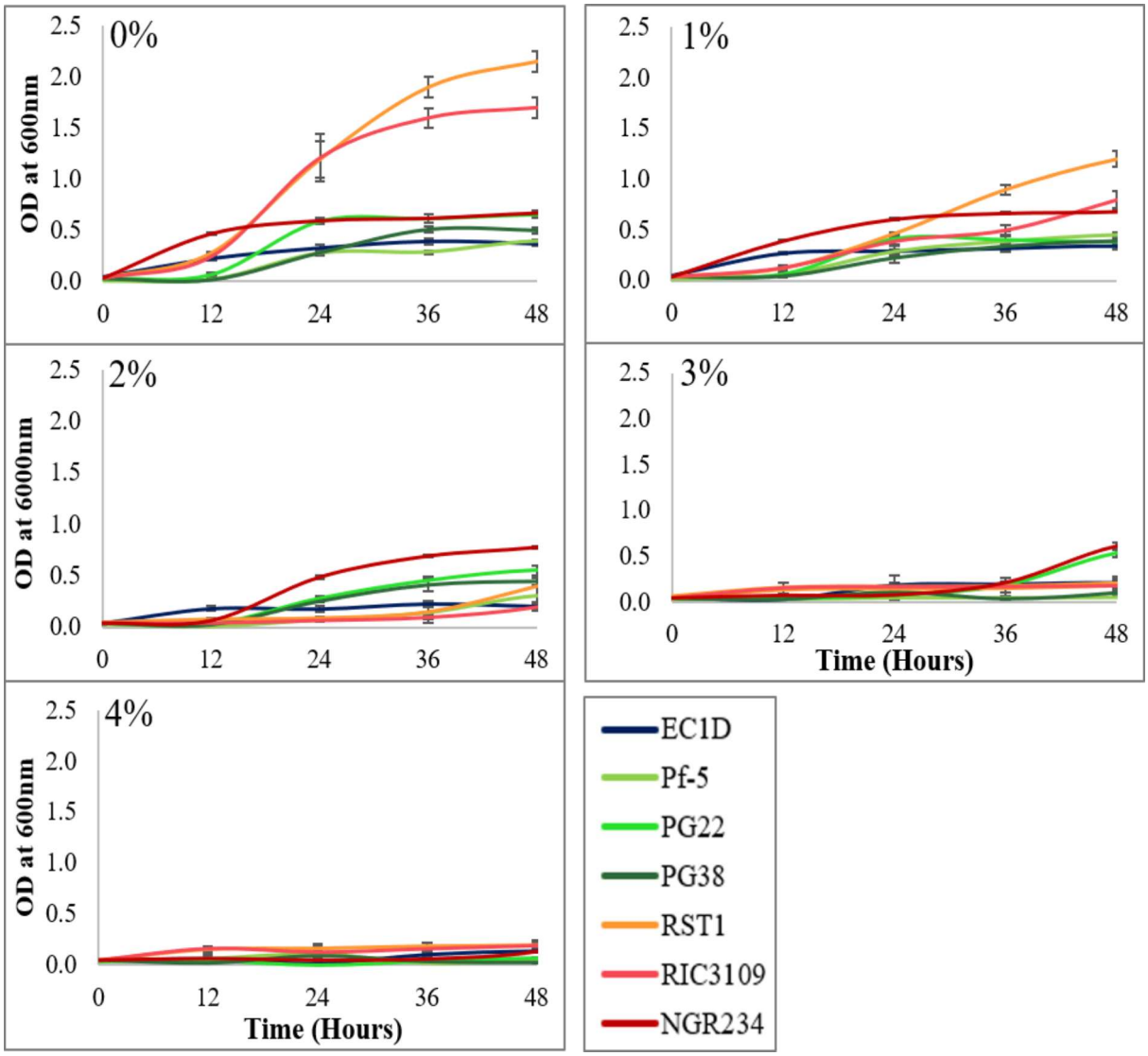

Figure 3.5: Growth activity of individual PGPR strains in TRP medium under salt stress.

3.3.2.1. Growth of PGPR consortia under phosphate deficiency and salt stress

In consortia, growth reached stationary phase before 24h in most of the consortia (Figure 3.6). Delay in growth increased with increasing salt stress in all the combinations however, the final growth density was higher as compared to control sets until 3% salinity. The consortia having NGR234 were least affected by salt stress at all salt concentrations.

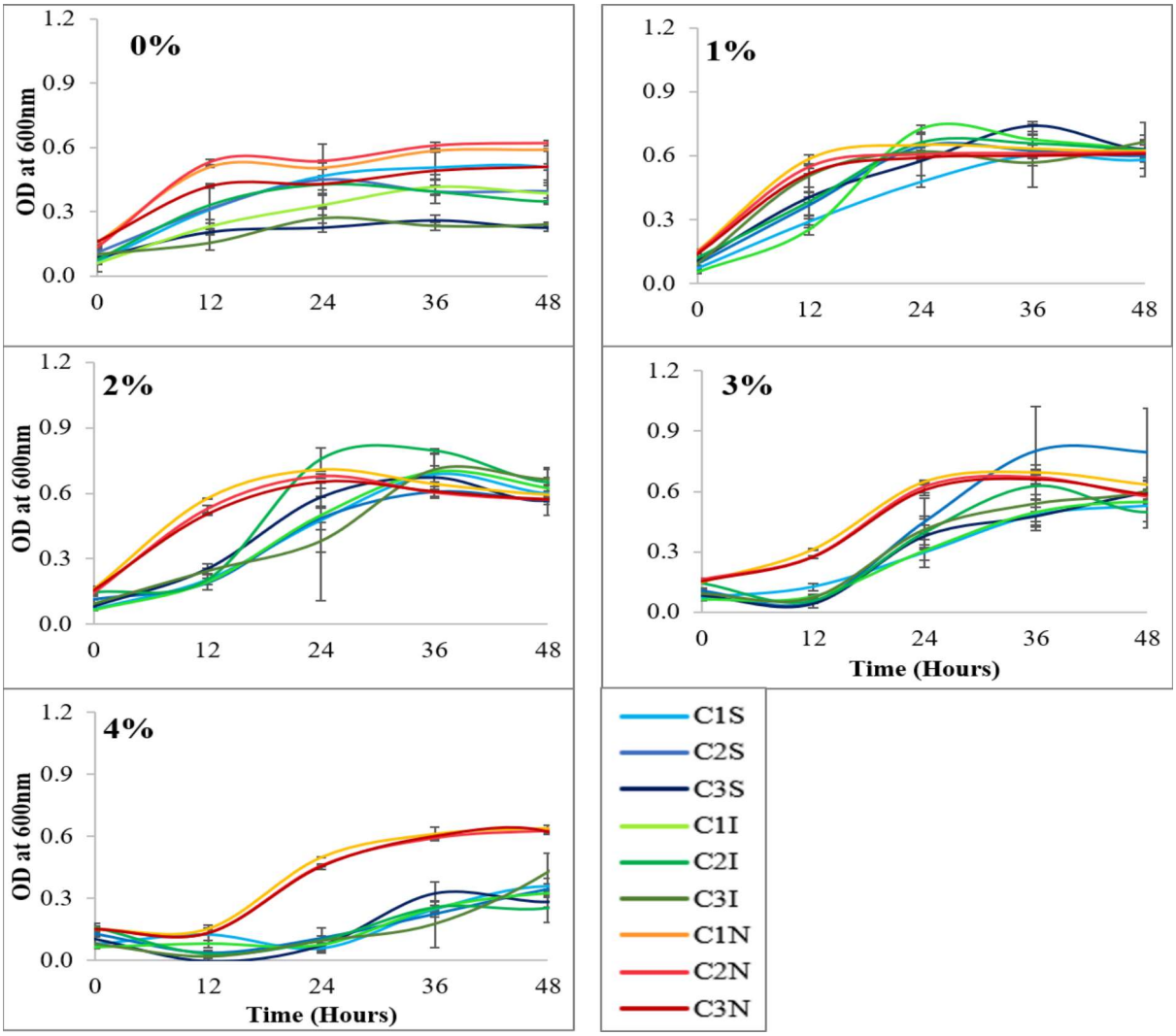

Figure 3.6: Growth of PGPR consortia in TRP medium under salt stress.

3.3.3. Semi-quantitative estimation of individual bacterial populations using genus specific primers

Figure 3.7 shows PCR amplification results using specific primers. Increase in band intensity with time was observed in most of the consortia for all the strains.

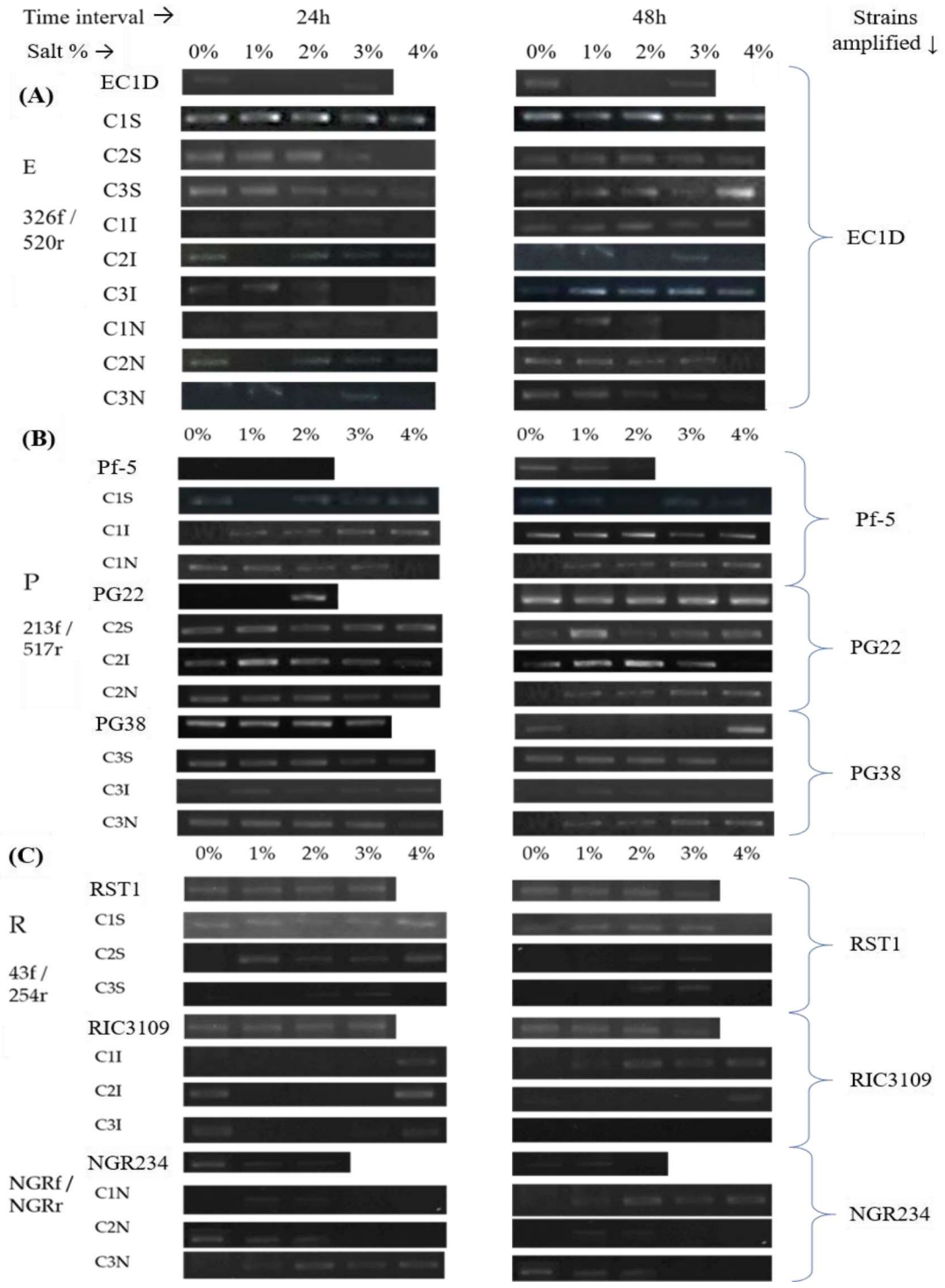

Figure 3.7: PCR amplification of 16S rRNA genes from PGPR consortia using genus specific primers for (A) EC1D, (B) Pseudomonas, (C) Rhizobium sp. and NGR234 at 24h and 48h time periods. The first row of each section shows individual strains while the remaining are consortia containing the particular strain.

The Figure 3.8 shows the Image Lab software (v 6.1) analysis of the band intensities of PCR amplification from the images shown in Figure 3.7. When grown individually, the population of EC1D (as indicated by 16S rRNA amplicon band intensity, Figure 3.7A) under P-starved condition was increased, while salt stress decreased the growth rate, the population at 3% salinity was found to be enhanced by 5-folds (Figure 3.8 A). In consortium, EC1D had substantially high population density in consortia C1S, followed by those in combination with RST1 and RIC3109. It showed stability to survive in the dually stressed conditions. PG22 and PG38 stimulated maximum population density for EC1D in consortia.

Among *Pseudomonas* sp. PG22 and PG38 showed population density in P-deficiency and at all salt concentrations when grown individually (Figure 3.7 B), which co-related to the high P-solubilization observed in these consortia under salt stress (Table 3.3). In control set (individual PGPR), delayed appearance of detectable bacterial population was observed, while only PG22 showed growth in 4% salinity (Figure 3.8 B). In consortia all the pseudomonads showed abundance at 4% NaCl. The high bacterial population in C1I was relatable to the highest P-solubilization activity in this consortium.

The strains RST1 and RIC3109 showed similar population density in P-deficiency and all salt stress concentrations when grown individually (Figure 3.8 C). Among consortia C1S showed high bacterial population at salt concentrations (Figure 3.7 C). In some consortia (C2S, C3S, C2I) salt stress induced increase in bacterial growth, these consortia also showed high P-solubilization activity under salt stress (Table 3.3). Band analysis revealed high bacterial population under salt stress for rhizobial strain RST1. The bacterial population for NGR234 was negligible and this strain did not show bacterial growth under stress (Figure 3.3)

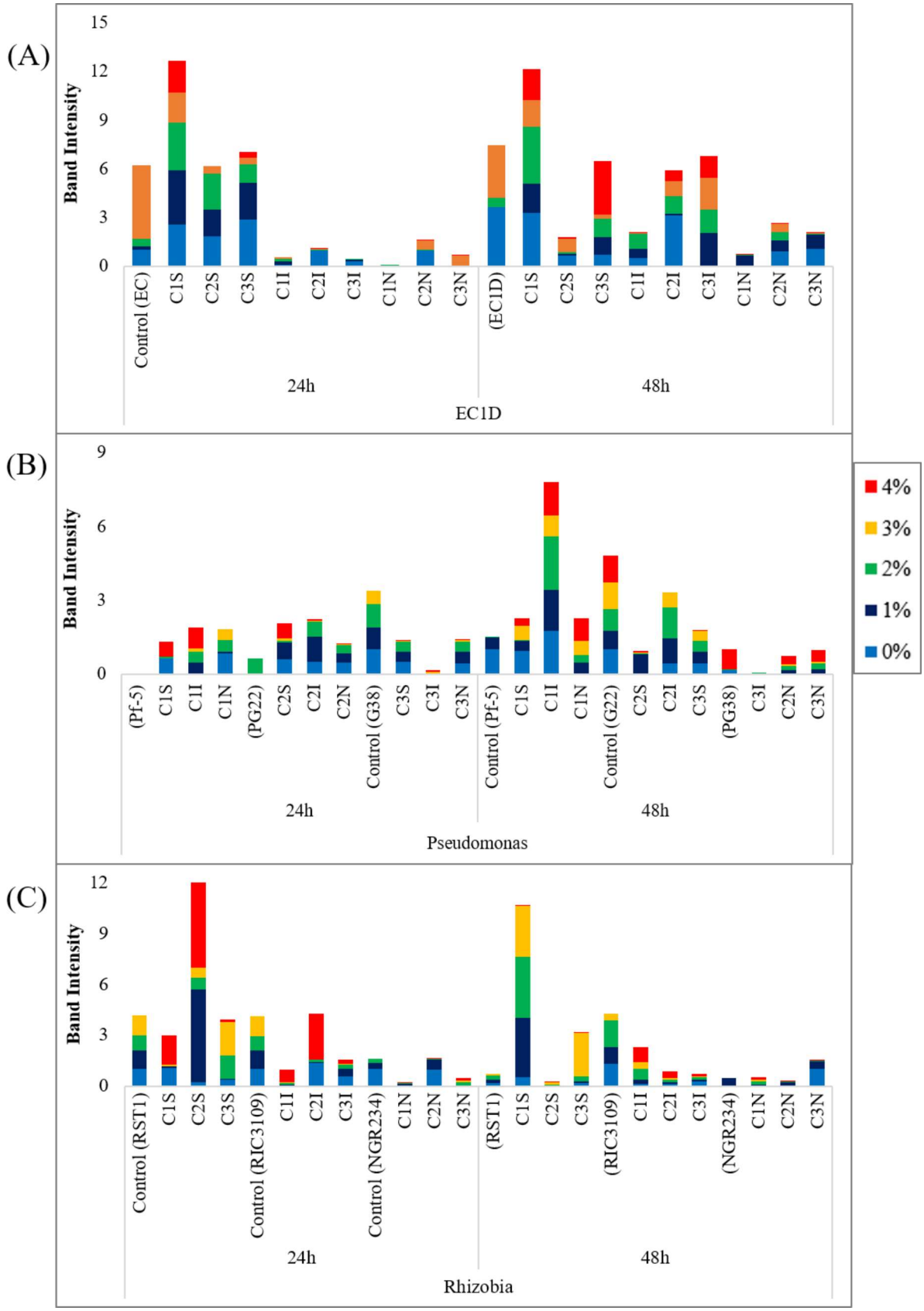

Figure 3.8: Semi-quantitative analysis of population density in (A) EC1D, (B) Pseudomonas spp. and (C) rhizobia in PGPR consortia under salt stress, compared to individual strains (in parenthesis) grown in same conditions as control.

3.3.4. Population dynamics of PGPR consortia in rich medium under NaCl stress

Here the qPCR approach was used to assess the PGPR populations in consortia during growth in rich medium under the influence of salt stress.

3.3.4.1. Bacterial growth and survival in environmental stress using NaCl as the stressor

The PGPR strains and consortia were subjected to increasing concentrations of NaCl in the LB broth medium. Figure 3.9 shows the growth curves for each individual PGPR. As seen in the earlier observations, (Chapter 2) EC1D demonstrated maximum tolerance towards growth till 5% salinity was reiterated. Strain Pf-5 showed tolerance till 4% stress. Although PG22 and PG38 showed higher optical density at low salt concentrations, these strains barely tolerated 4% NaCl stress. Pseudomonad growth was absent at 5% salinity. The *Rhizobium* spp. survived only 3% salinity while NGR234 could not tolerate 2% salt stress even when grown in favoured conditions (tryptone yeast medium).

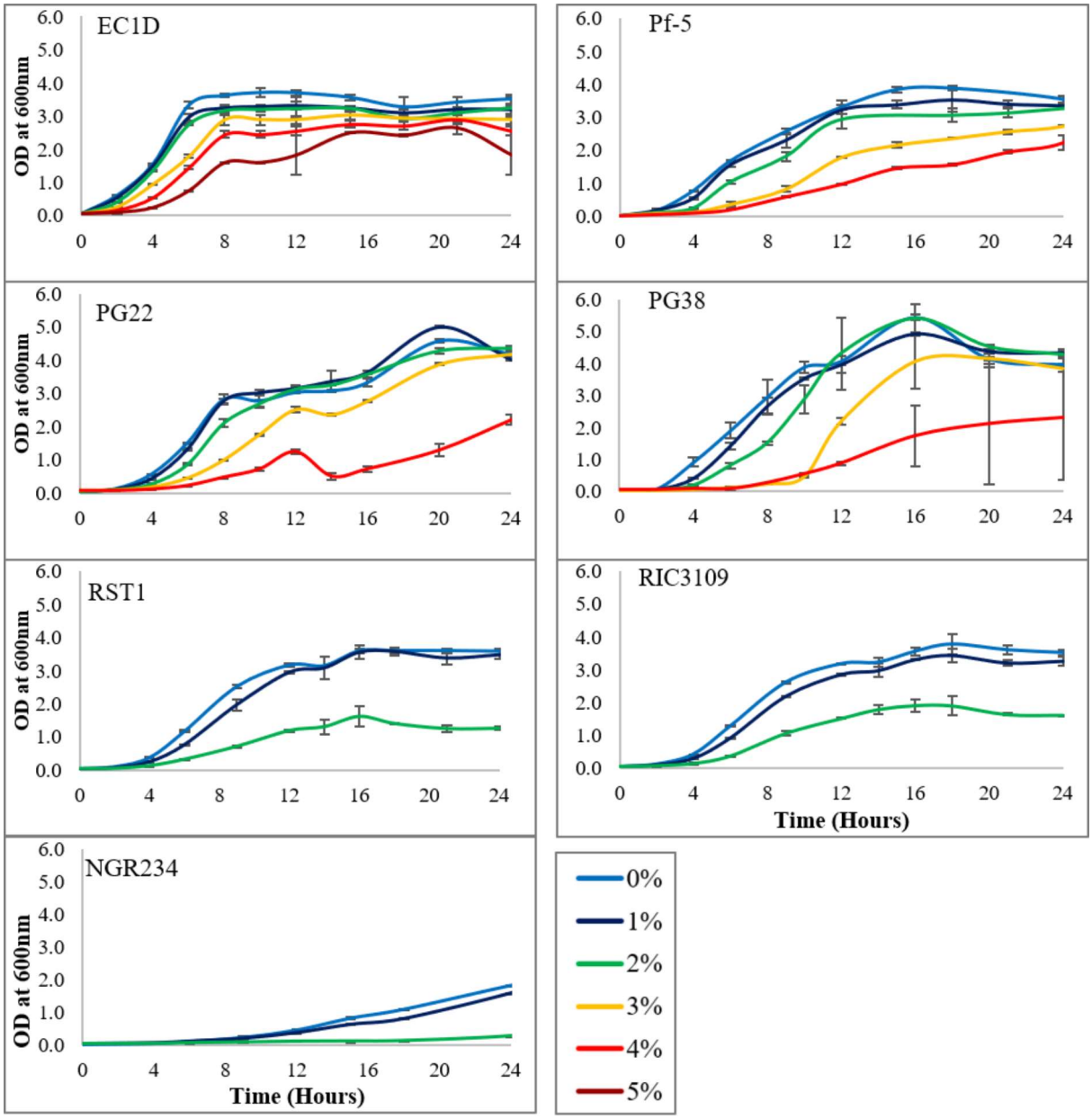

Figure 3.9: Growth of bacterial strains under salt stress.

3.3.4.1. Effect of Salt stress on the bacterial consortia

As observed in the above experiments, consortia formation has led to enhanced PGPR effect under salt stress. These bacteria when grown in consortium showed remarkable increase in their tolerance towards salt stress which was enhanced up to 5% NaCl concentration in all the consortia. Similar growth curves were observed among the consortia at each salt concentration (Figure 3.10). The dotted lines indicated maximum growth at 0% salinity which decreased gradually beyond 1% NaCl concentration. The consortium having PG38 showed highest growth density at all salt concentrations, followed by PG22 consortia.

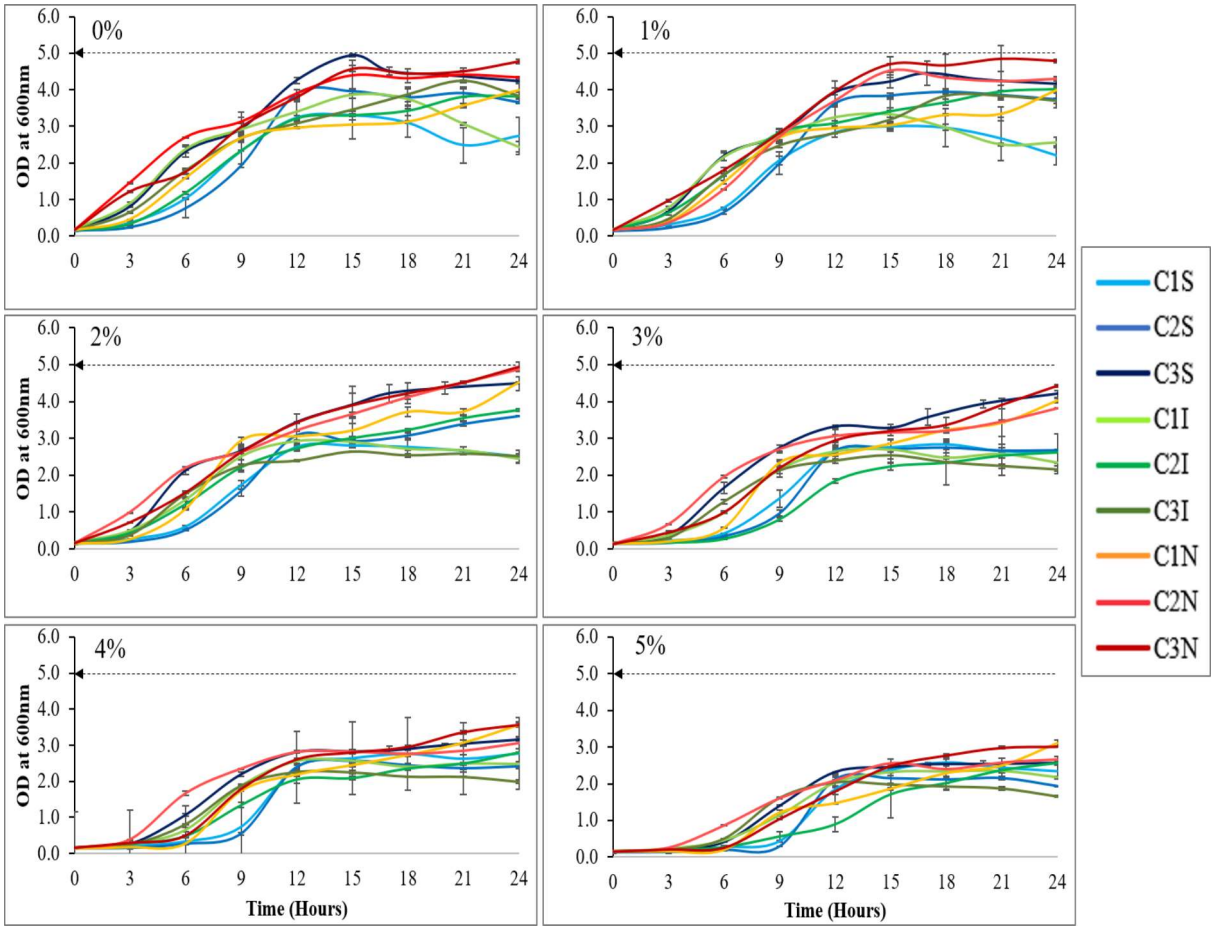

Figure 3.10: Effect of salt stress on bacterial consortia Growth of bacteria in consortia under salt stress. the dotted lines represent maximum growth observed in consortium in no salt control as a control for decrease in growth density.

3.3.4.2. Population density in bacterial consortia based on copy number values

Since EC1D was common component in all the consortia, the major variance among consortia performance was difference in pseudomonads and rhizobia strains. Based on the standard graphs (Appendix II), bacterial population was estimated, as the copy number from the Ct values obtained in QPCR analysis, for each strain in the consortia. Growth of EC1D was enhanced in most of the consortia while salt stress (1% NaCl) also induced growth in consortia (C1S, C1N, C2I, C3I) as compared to no salt control (Table 3.4). In consortia with NGR234 population density was maintained till high salt concentrations, while the *Rhizobium* spp. had varied effect on EC1D growth depending on the pseudomonad component and salt stress. With the exception of C2I at 4% salinity, most of the consortia treatments show growth enhancement in EC1D under salt stress as well. At 5% NaCl, an ~10-fold increase in the bacterial growth was observed in all consortia with PG38 under control and salt stress condition.

Table 3.4: Copy number of Strain EC1D from consortia under salt stress at 12h and 24h time period.

Hours	Salt %	Copy number									
		EC1D	C1S	C2S	C3S	C1I	C2I	C3I	C1N	C2N	C3N
12h	0	9.57E+05	3.05E+06	2.68E+07	5.85E+07	8.22E+06	6.69E+06	1.10E+06	2.61E+06	5.68E+07	1.27E+07
	1	6.46E+05	4.72E+07	7.34E+06	9.67E+07	7.84E+06	3.47E+06	4.78E+07	1.34E+07	3.69E+07	2.39E+07
	2	6.76E+05	1.15E+07	1.91E+07	1.36E+07	7.92E+06	2.31E+06	8.24E+07	2.44E+07	4.49E+07	1.49E+07
	3	1.57E+06	4.08E+06	6.41E+06	2.87E+07	7.32E+06	1.16E+06	3.91E+07	2.25E+07	2.56E+07	7.23E+07
	4	1.60E+06	4.69E+06	8.81E+06	1.06E+08	5.50E+06	9.25E+05	3.18E+07	2.12E+07	4.72E+07	1.35E+07
	5	2.90E+06	3.26E+07	7.82E+06	3.53E+07	1.30E+07	1.19E+06	1.19E+07	8.52E+06	3.53E+07	1.23E+07
24h	0	6.26E+05	8.60E+06	1.36E+07	2.51E+07	1.34E+07	1.28E+06	2.89E+07	7.18E+06	1.43E+07	1.57E+07
	1	6.46E+05	4.95E+07	7.98E+06	1.22E+07	1.09E+07	1.91E+07	5.59E+07	2.43E+07	1.89E+07	3.76E+07
	2	6.06E+05	4.05E+06	7.21E+06	1.91E+07	3.33E+06	2.27E+07	7.89E+07	6.32E+07	5.31E+07	1.06E+07
	3	9.30E+05	3.73E+06	8.03E+06	2.09E+07	1.54E+07	8.12E+06	8.32E+07	4.76E+07	8.39E+07	2.64E+07
	4	1.26E+06	6.37E+06	8.22E+06	2.65E+07	7.02E+06	3.87E+06	1.21E+08	2.08E+07	4.17E+07	1.38E+07
	5	1.94E+06	6.63E+06	7.71E+06	2.05E+07	1.35E+07	5.88E+06	1.07E+08	4.28E+07	3.22E+07	5.02E+07

Among pseudomonads, in consortia with NGR234 the population of pseudomonads was enhanced to ~10-folds in most salt concentrations (Table 3.5). The pseudomonads in consortium showed growth at 5% salinity where individual strains failed to survive. PG22 and PG38 showed maximum increase in population in consortium under salt stress compared to individual strains. It was also observed that the population density of pseudomonads was much higher in comparison to EC1D and *Rhizobium* spp. Although delayed growth was also observed at higher salt stress.

Table 3.5: Copy number of Pseudomonas spp. from the different consortia under salt stress at 12h and 24h time period.

Hours	Salt %	Copy number											
		Pf-5	PG22	PG38	C1S	C2S	C3S	C1I	C2I	C3I	C1N	C2N	C3N
12h	0	2.53E+11	2.00E+11	2.57E+11	2.01E+06	7.45E+11	1.13E+13	1.56E+06	1.04E+13	8.84E+09	6.98E+11	2.71E+12	1.39E+11
	1	2.73E+11	1.43E+11	1.88E+11	6.28E+06	1.91E+11	3.28E+12	3.59E+05	1.71E+12	1.90E+11	1.60E+12	2.89E+12	2.51E+13
	2	1.76E+11	1.40E+11	1.02E+11	5.13E+05	1.40E+11	7.94E+10	1.18E+06	6.50E+12	9.73E+09	9.59E+11	5.18E+11	3.47E+12
	3	3.70E+11	6.55E+10	5.41E+10	6.11E+05	2.47E+09	1.30E+11	5.08E+05	4.19E+09	4.08E+10	8.86E+11	1.12E+11	4.58E+12
	4	9.86E+10	1.04E+11	1.84E+11	1.26E+06	1.76E+09	4.42E+11	4.29E+05	2.67E+09	9.56E+10	1.72E+11	9.74E+10	2.41E+11
	5	0.00	0.00	0.00	6.22E+06	2.34E+08	7.32E+10	6.12E+06	6.92E+11	9.15E+09	9.27E+10	1.07E+11	5.85E+11
24h	0	2.35E+11	1.38E+11	1.10E+11	2.09E+06	2.98E+11	1.91E+12	3.57E+05	1.03E+11	2.54E+12	3.60E+12	9.75E+12	8.29E+12
	1	1.21E+11	1.10E+11	6.48E+10	2.56E+07	1.94E+10	4.03E+11	6.06E+05	4.14E+12	1.75E+12	3.61E+12	3.60E+12	1.37E+13
	2	2.92E+11	1.75E+11	1.33E+11	3.05E+06	7.15E+08	9.97E+10	3.03E+05	3.46E+12	2.25E+11	2.88E+12	2.43E+12	2.43E+12
	3	2.37E+11	6.36E+10	2.58E+11	1.88E+06	3.11E+09	4.07E+10	1.56E+06	1.79E+12	2.87E+10	1.23E+12	1.58E+12	1.12E+12
	4	1.21E+11	3.84E+10	1.09E+11	6.60E+05	1.97E+08	1.24E+11	5.77E+05	6.17E+12	3.00E+09	1.46E+11	8.43E+11	4.24E+11
	5	0.00	0.00	0.00	1.68E+06	3.75E+08	3.60E+09	1.75E+06	2.29E+13	5.03E+09	2.23E+11	1.61E+11	4.15E+11

The *Rhizobium* sp. showed decreased population under salt stress. NGR234 showed increased population in consortia in no salt control as well as 1% salinity but only during the early growth phase (12h). NGR234 showed high population in combination with pseudomonads in

no salt control (Table 3.6) while increasing salinity led to decrease in cell count. The population density in *Rhizobium* spp. was reduced by ~E2-E3-folds in all the consortia but growth remained constant even at high salt concentrations.

Table 3.6: Copy number of Rhizobia from the different consortia under salt stress at 12h and 24h time period.

Hours	Salt %	Copy number											
		RST1	RIC3109	NGR234	C1S	C2S	C3S	C1I	C2I	C3I	C1N	C2N	C3N
12	0	1.14E+07	9.28E+06	1.74E+12	5.27E+04	3.92E+04	5.74E+04	1.34E+05	4.93E+04	6.25E+02	1.31E+11	1.55E+13	7.12E+18
	1	1.08E+07	1.20E+07	1.17E+12	9.41E+04	1.30E+04	5.76E+04	1.59E+05	3.55E+04	2.00E+04	5.93E+11	1.11E+12	1.51E+17
	2	6.83E+06	8.07E+06	1.23E+12	7.85E+03	2.41E+04	1.14E+04	7.55E+04	3.06E+03	1.44E+04	6.17E+11	3.92E+10	1.69E+11
	3	0.00	0.00	0.00	1.63E+04	1.02E+04	3.66E+04	6.20E+03	1.00E+04	8.49E+03	2.42E+11	3.11E+10	3.47E+13
	4	0.00	0.00	0.00	6.05E+03	1.73E+04	3.75E+04	3.82E+04	3.33E+04	1.74E+04	4.96E+10	3.45E+10	2.61E+10
	5	0.00	0.00	0.00	2.22E+04	2.45E+04	8.23E+04	1.79E+04	3.49E+03	1.85E+04	2.38E+11	2.93E+11	2.51E+12
24	0	1.15E+07	9.15E+06	1.14E+12	9.74E+04	2.78E+04	7.09E+04	7.09E+04	6.05E+04	6.88E+03	1.06E+20	1.90E+21	6.04E+12
	1	1.01E+07	7.74E+06	1.17E+12	3.22E+05	2.60E+04	3.30E+04	1.03E+05	7.88E+04	1.11E+04	7.74E+11	1.13E+13	9.71E+11
	2	5.43E+06	3.88E+06	1.1E+12	1.20E+04	1.78E+04	8.34E+04	4.20E+04	3.51E+05	1.64E+04	7.20E+11	3.55E+11	1.50E+11
	3	0.00	0.00	0.00	8.74E+03	1.99E+04	4.44E+04	3.38E+04	1.74E+05	2.10E+04	1.68E+11	9.93E+11	2.61E+11
	4	0.00	0.00	0.00	1.41E+04	1.89E+04	1.98E+04	5.78E+04	3.52E+04	2.12E+04	5.04E+09	3.70E+11	7.07E+10
	5	0.00	0.00	0.00	1.21E+04	3.36E+04	1.16E+04	7.71E+04	1.72E+04	5.66E+03	1.83E+11	5.34E+11	6.84E+11

Figure 3.11 shows the composition of each consortium in terms of the population of each of its individual members. It is interesting to note that EC1D population was largely stable in all the consortia at all salt concentrations. Among the three members in consortia, the population of the rhizobia was most fluctuating depending on the combination of strains rather than on the basis of salt concentration indicating strain specific interactions driving the survival of rhizobia in the consortium. A good compatibility among the three members of consortia (in terms of balanced growth of all members) was seen in case of consortia with NGR234 (C1N, C2N, C3N). Consortia C1S and C1I had low number of all three members.

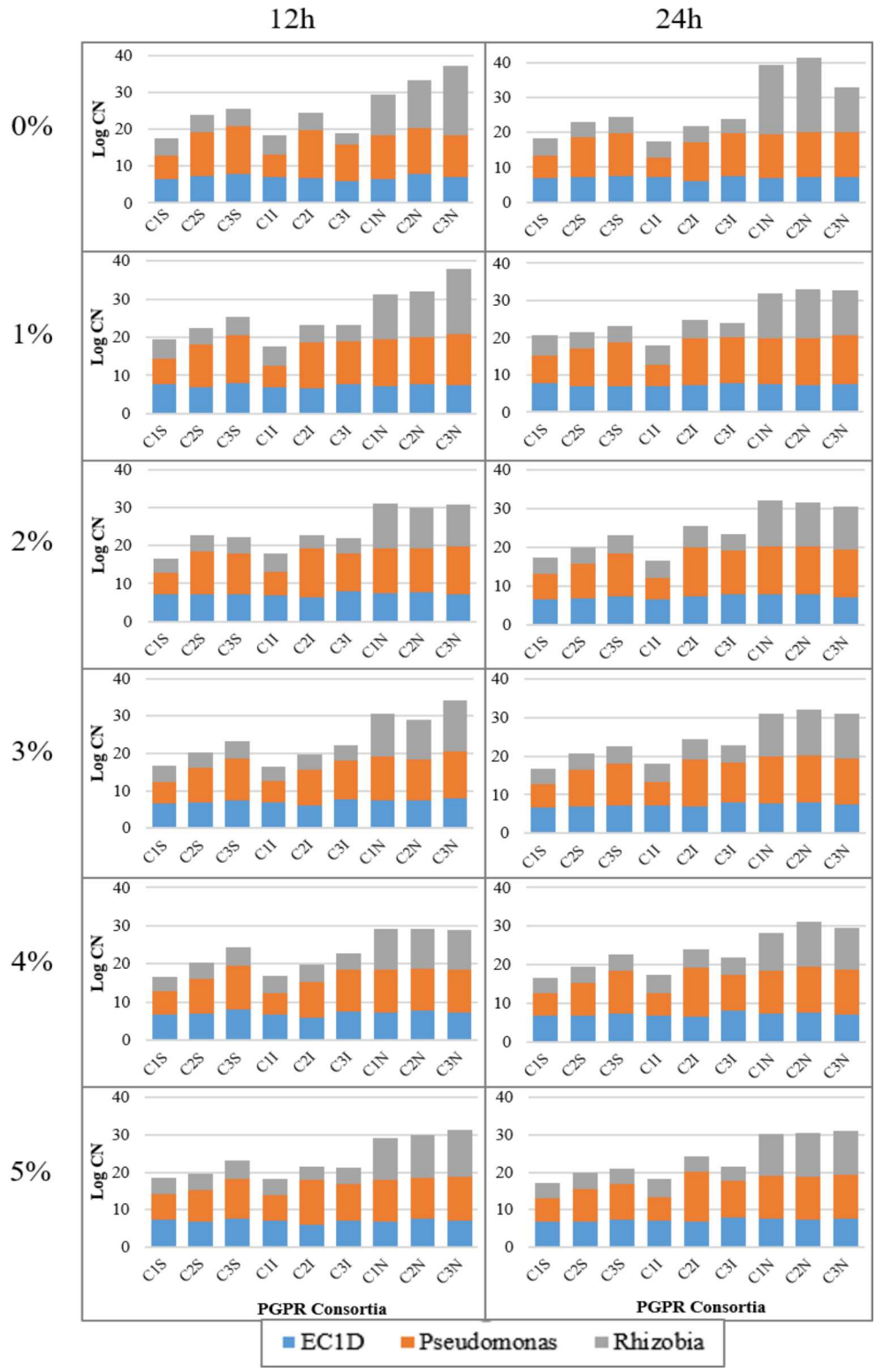

Figure 3.11: Log copy number (CN) values for each of consortia member in the respective consortium under increasing salt concentrations at 12h and 24h time period.

3.4. Discussion

Using rock phosphate as the sole source of phosphorous in the medium in buffered conditions, the bacteria were studied for their ability to solubilize this phosphate so as to release the soluble inorganic form which can be easily taken up by the plants (Buch et al., 2008).

Under saline stress, EC1D gave highest P-solubilization which was similar to the studies reported by (Sharma et al., 2005) in which 890µmol/L P-release was reported in *E. asburiae* when the medium reached to pH 5 in presence of glucose as the sole carbon source, EC1D produced ~900µmol/L P-release within 12h into the experiment for the pH to reach the same limit (data not shown). Jiang et al., (2018) have reported *Enterobacter* sp. with highest P-solubilization efficiency showing >200mg/L (~1.4mmol/L), which was slightly higher than that observed in the EC1D.

Decrease in P-solubilization efficiency under salt stress was commonly observed among most of the PGPR and consortia under the present study. (López et al., 2020) have reported *Talaromyces islandicus* as PGPR with efficiency to solubilize rock phosphate (~490mg/L P-release), however it was reduced to 45% efficiency at 3% salt stress. However, Pf-5 and a few consortia also showed increased P-solubilization at higher salt concentration (2% NaCl). These results corelated to the study reported by Tchakounte et al. (2020) where an increase in P-solubilization from ~127mg/L to ~193mg/L was observed at 4% salinity in presence of tricalcium phosphate as the sole P source.

P-solubilization at 4% salinity under high salt stress (Chapter 2) and high salt tolerance in growth conditions were observed only in EC1D, which can be attributed to the high salt tolerance ability and P-solubilization efficiency of the consortia. Kumar et al. (2021) have shown high phosphate solubilization by pseudomonad strains which was higher when in consortium demonstrating an ~2-fold increase in the P-solubilization. The pseudomonads PG22 and PG38 which showed high P-solubilization efficiency also showed similar results in consortium under salt stress. Although most of the consortia demonstrated pseudomonad dependent demonstration of PGPR traits, the consortia C1N, C2N and C3N showed the low P-solubilization compared to other consortia and similar to NGR234 grown individually.

In the present study, highest P-release was observed in the consortia C1I, which showed high biofilm formation in no salt condition. The consortia having pseudomonads PG38 and PG22

in combination with RST1, followed by RIC3109, have shown highest phosphate solubilization under salt stress. As discussed before, high phosphate solubilization in consortia comprising pseudomonads along with other PGPR has been associated with biofilm formation and (Behera et al., 2021) and siderophore producing traits (Ahmed & Holmström, 2014).

The high optical density observed among *Rhizobium* spp. grown individually can be attributed to exopolysaccharide (EPS) production under stress, since the initial population was maintained till 3% salinity even though the bacteria was unable to produce P-solubilization (Figure 3.8C). Amaya-Gómez et al. (2020) have also reported the biofilm forming ability in *Rhizobium* spp. to be influenced by the P-solubilization activity. Under P-deficiency the individual strains showed high growth density up to 48h in all the strains, however, PCR amplification analysis did not co-relate to this observation. Biofilm production can be a possibility as a result of the dual stress while the bacterial strains were not able to survive. Similar results were also observed for PG22 where bacterial population was negligible at 12h however high optical density was observed in individual strains. All of these strains and most of the consortia under were able to show high biofilm production under salt stress. (Bochuan et al., 2014) have reported dual culture consortium in which *P. putida* stimulated increased biofilm formation in *S. oneidensis* which enhanced the bioremediation activity for breakdown of Azodye. The biofilm formation was elucidated by fluorescence tagging in both the strains.

The semi-quantitative PCR method is highly useful in studies related to community shifts with respect to fluctuations in environmental factors (Props et al., 2017; Wilhelm et al., 2015). In the present study, the conserved 16S ribosomal RNA partial gene sequences were used for primer designing with genus-based specificity, since each consortium was comprised of one strain from 3 different genera. The two techniques were primarily utilized for relative quantitation of bacterial strains in the consortia under different stressed conditions when compared to individual strains. It provides high specificity, with less time consumption and low economic expense (Alaylar et al., 2020). The primers can be used for any species of the said genus and the amplified products were small enough to be applicable in qPCR analysis for selective amplification in mixed consortia as well.

Establishing link between consortia composition to function in combination with quantitative PCR could thwart the major challenge as an untargeted approach and allow absolute taxon quantification to be computed as well (Widder et al., 2016).

Timeline based selection using 16S sequencing technique provides optimal selection of microbial community (Wright et al., 2019), while it can also help to identify variation in microbial diversity based on changes in the environment (Pei et al., 2021). However, in the present study, a targeted approach could be applied since the microbial consortia were composed of known bacterial samples. Understanding the dynamics of a consortium has a wide range of applications in fields ranging from food science to waste water treatment. Hussain et al., (2021) showed the presence of several pathogenic strains was only found during initial stages and were eliminated later on, proving the efficacy of food processing techniques, based on 16S sequencing for microbial community analysis.

Analysis of the bacterial population for each consortia member revealed a major impact of consortia development by growth enhancement of EC1D and improving salt tolerance in *Rhizobium* spp. Choure et al. (2012) have reported positive interaction in a multispecies microbial consortium having *Pseudomonas sp, Sinorhizobium* sp. and *Azotobacter* sp. where spectrophotometric techniques were utilized to analyse the growth of individual strains in presence of cell-free supernatants from *Pseudomonas* strains, and was found to be at par compared to individual strains. In the present study, pseudomonads showed high population density in consortia as compared to other strains which can also explain the dominance of *Pseudomonas* spp. on the PGPR trait demonstration as was discussed in the previous Chapter (2).

Among pseudomonads, Pf-5 in consortia C1I showed ~2-fold growth enhancement, even though individually it was unable to grow under high salt stress (absent beyond 1% NaCl) (Figure 12). These results are in line with the study reported by Sanchez-Gorostiaga et al., (2019), where growth stimulation in *P. polymyxa* was enhanced when grown in cell free supernatant from other species compared to monocultures. Zupančič et al., (2018) have also reported high potential in the *Pseudomonas* strains to co-exist with bacteria from other genera in a consortium. The least effect of salt stress on bacterial population was observed among the consortia having NGR234 as the rhizobial component which continued to stable density till 4% salt stress, even though individual strains did not survive at this concentration.

Another study reported by Deng & Wang, (2016), showed that the complexity of substrate determined the synergistic growth in three-member bacterial consortia. It was observed that the bacteria attained synergistic growth to degrade a complex substrate such as lignocellulose, while in the presence of a labile substance such as glucose, bacterial competition for the limited nutrient led to antagonistic interaction. The bacterial growth measurements were performed by simple spectrophotometric technique. Thus, it can be said that the stress factors stimulate consortial synergy which can explain the higher salt tolerance among the consortia observed in the present study.

Summarizing, in this chapter it was observed that consortia development provided a stability to bacterial growth. Although most of the strains showed delayed growth due to salt stress, some of the strains were also observed to show increased tolerance for growth at higher salt concentrations which was absent during individual growth experiments. P-deficiency and salt stress led to synergistic association among the consortial members which facilitated enhanced population density and stress tolerance.

Application of molecular techniques provide a more accurate means for enumeration of bacteria to evaluate the PGPR traits under stressed conditions and for analysing the actual contribution of each group of bacteria in a consortium, which might be responsible for the heightened response. It can help provide more information regarding dominant species and synergistic associations in rhizosphere for microbial sustenance as well as plant growth under salt stress. Thus, bacterial growth under salt stress and in consortium provided a deeper understanding of the consortial association among the strains which confirmed the ability of these consortia to survive for longer durations under salt stress and can be helpful for alleviation of stress and growth promotion in plants as well.