Chapter 3

Determining the MPS ability of *Rhizobium* strains by incorporating *pqqE* gene and *Acinetobacter calcoaceticus pqq* gene cluster.

3.1: Introduction:

The source of soil nitrogen is the atmosphere where nitrogen gas occupies about 79% of the total atmospheric gases. Living organisms that are present in the soil have profound effect on transformation, which provides food and fiber for an expanding world population. It is stated that nitrogen returned to the earth every year, microbiologically is of the order of 139 x 10^9 kg of which about 65% (89 x 10^9 kg) is contributed by nodulated legumes (Rashid et al., 2008; Shridhar, 2012). Symbiotic nitrogen fixation by rhizobia in legumes has a profound impact upon agriculture and human endeavour. Phosphorus has a key role in the energy metabolism of all plant cells and particularly for nitrogen fixation in legume crops (Israel, 1987; Erman et al., 2009). Plant available nitrogen is present in millimolar amounts, while the plant available phosphorous is usually in micromolar amounts (Anthony et al., 2009).

Nitrogen fixation by *Rhizobium* in field depends on soil P levels and dual inoculation of *Rhizobium* with PSMs improves crop productivity (Gull et al., 2004; Elkoca et al., 2007; Valverde et al., 2007; Kumar and Chandra, 2008). Co-application of *Rhizobium* isolates and phosphate solubilizing microorganisms in soils with low available phosphorus improved yield production of mung beans (Perveen et al., 2002). Higher number of nodules and dry weight were seen in soybean and alfalfa under co-inoculation with *Rhizobia* strains and phosphate solubilizing *Pseudomonas* strains (Rosas et al., 2006). Increased nodulation ability of *Rhizobium* isolates on bean plants was observed as a result of phosphate solubilizing PGPR co-application (Remans et al., 2007). Dual inoculation of soybean seeds with *Bradyrhizobium japonicum USDA110* and PSB significantly increased plant height, nodule fresh weight per plant, number of pods per plant, number of seeds per pod and per plant, seed yield, total N, and P compared to the other treatments (Argaw, 2012). *Rhizobium* and phosphate solubilizing bacteria significantly increased yield of faba bean and a synergetic effect was observed when the two types of microorganisms were combined (Rugheim and Abdelgani, 2012).

Phosphate solubilizing *Rhizobium* species showing high ability to nodulate and fix nitrogen was isolated from common bean *Phaseolus vulgaris* (Abril et al. 2003). Increase in growth of maize and lettuce was observed with P- solubilizing

Rhizobium leguminosarum (Chabot et al., 1996). Phosphate solubilizing *Mesorhizobium meditteraneum* increased growth, N, P, Ca, Mg, and K in chick pea and barley (Peix et al., 2001). Mineral phosphate solubilisation (MPS) is mediated by organic acids such as gluconic, 2-ketogluconic, citric, lactic, oxalic and succinic (Hwangbo et al., 2003; Qureshi et al., 2011; Archana et al., 2012). Gram Negative bacteria utilize direct oxidation glucose pathway to produce gluconic and 2-ketogluconic acids (Krishnaraj and Goldstein 2001). Conversion of glucose to gluconic acid is facilitated by pyrolloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) (Buch et al. 2008; Castagno et al., 2011). Enzyme is present in the outer face of the cytoplasmic membrane, so acids are formed in the periplasmic space, with the resultant acidification of this region and, ultimately, the adjacent medium as well (Babu-Khan et al. 1995 ; Shashidhar and Podile 2009). Gram Negative bacteria like *E. coli*, *Azospirillum* , *Herbaspirillum* show presence of apoGDH and lack *pqq* genes ; while *Acinetobacter, Gluconobacter, Pseudomonas, Erwinia* species possess both *pqq* and *gcd* genes. Number of genes required for PQQ synthesis varies from species to species (Choi et al., 2008). PQQ biosynthesis is not completely understood but a putative biosynthetic pathway has been proposed on the basis of the functions of conserved genes in bacteria (Puehringer et al., 2008). *Klebsiella pneumonia* possesses *pqqABCDEF* genes which are involved in PQQ biosynthesis (Meulenberg et al., 1992). *pqqA* gene encodes for 23-24 amino acid polypeptide which is a substrate for a set of enzymes modifying the glutamate and tyrosine residues leading to PQQ formation (Goosen et al. 1992; Meulenberg et al. 1992; Velterop et al. 1995). PqqB belongs to metallo-β-lactamases and has been suggested to help in the transport of PQQ into periplasm (Velterop et al., 1995). PqqC is a cofactor less oxidase, activates oxygen, and catalyzes the final step of ring closure reaction (Magnusson et al., 2004). PqqD has been shown to interact with PqqE which possesses reductively cleavage activity of S-adenosyl methionine to form 5' deoxyadenosine and methionine (Wecksler et al., 2010). PqqE catalyzes the first step of linking glutamate and tyrosine residues of PqqA peptide. Additionally, PqqF, G, H, I, J, K and M are found in some bacteria possessing putative Zn dependent peptidase, non catalytic subunit of peptidase, transcriptional regulator, aminotransferase, cytosolic protein, DNA binding and prolyl oligopeptidase, respectively (Choi et al., 2008).

Expression of *Erwinia herbicola pqqE* gene alone in *E. coli* HB101 and *Azospirillum* resulted in secretion of gluconic acid by converting apoGDH to active form (Liu et al., 1992; Vikram et al, 2007). Incorporation of *Rahnella aquatilis pqqED* genes in *E. coli* resulted in gluconic acid secretion (Kim et al., 1998). The mechanism of PQQ biosynthesis either in *pqqE* or *pqqED* gene transformants of *E. coli* is not clear as *pqqABCDE* genes are necessary for PQQ biosynthesis (Choi et al., 2008; Shen et al., 2012). *E. coli* was genetically modified by cluster of *pqq* genes from different bacterial species to confer MPS ability (Goosen et al., 1989; Meulenberg et al., 1990; Khairnar et al., 2003; Yang et al., 2010; Mounira et.al., 2013). Out of five phosphates solubilizing bacteria isolated from naturally colonizing limonitic crust, three isolates secreting gluconic acid contained *gcd* and *pqq* genes (Perez et al., 2007). *pqqE* and *gcd* gene was detected in gluconic acid secreting sunflower colonizing phosphate solubilizing *Enterobacter sp.* Fs-11 (Shahid et al., 2012).

Bacteroids and free living form of *R. leguminosarum* oxidized glucose to gluconic acid when supplemented with PQQ (Van Schie et al., 1987). Gluconate production and GDH activity was observed in *R. leguminosarum*, *R. etli*, and *B. japonicum* strains when grown in medium containing PQQ, whereas *S. meliloti* 102F34 showed holoenzyme synthesis and gluconate production in absence of PQQ (Boiardi et al. 1996). PQQ synthesis in *S. meliloti* RCR2011 is constitutive while *R. tropici* CIAT899 was unable to synthesize it (Bernardelli et al., 2001). This shows that PQQ synthesis in *Rhizobium* species containing apoGDH is strain dependant. GDH mutant of *S. meliloti* showed impaired symbiotic phenotype and altered nodulation efficiency and competition ability relative to the wild-type strain (Bernardelli et al., 2008). *Rhizobium* species like *R. leguminosarum* and *M. loti* lack *pqq* but shows presence of *gcd* genes, while both the genes are found to be present in species like *B. japonicum* and *S. fredii*.

3.1.1: Rational of study

Bradyrhizobium japonicum USDA110, *M. loti* MAFF030669 *and S. fredii*

NGR234 contain apo GDH enzyme. *M. loti* does not possess *pqq* genes while *B. japonicum and S. fredii* NGR234 possess *pqq* genes which encode for the cofactor of GDH for gluconic acid production through direct oxidative pathway. Wild type *B. japonicum, M. loti and S. fredii* NGR234 secretes very low amount of organic acid and thus does not show MPS ability. Thus, the present study investigates the effect of incorporation of *E. herbicola pqqE* gene and *A. calcoaceticus pqq* gene cluster in *B. japonicum, M. loti* and *S. fredii* NGR234for gluconic and PQQ secretion. These transformants were also monitored for MPS ability.

3.2 EXPERIMENTAL DESIGN

The experimental plan of work includes the following-

3.2.1: Bacterial strains used in this study

All wild type and genetically modified *E. coli* and *Rhizobium* strains used in this study are listed in **Table 2.1** the plasmids used in the present study and their restriction maps are given in **Fig. 2.1.** *E. coli* DH10B was used for all the standard molecular biology experiments wherever required.

Table 3.1: Bacterial strains used in this study

Plasmid/Bacterial strains	**Characteristics**	**Source/Reference**
***E. coli* strains**		
E. coli DH10B	Used to maintain plasmids for routine use	Invitrogen, USA
pBBR1MCS-2	Broad-Host-Range vector; Km^r	Kovach et al., 1995
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r (100µg/ml)	Hester et al., 2000
pJNK1	pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Wagh, 2013
pJNK5	pUCPM18, Gm^r (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus.*	Wagh, 2013
***Rhizobium* strains**		
Bradyrhizobium japonicum USDA110	Accession number NC_004463.1	NCBI
Mesorhizobium loti MAFF030669	Accession number NC_002678.2	NCBI
Sinorhizobium fredii NGR234	Accession number NC_012587.1	NCBI
***Bj* pUCPM18**	*B. japonicum* with pUCPM18, Gm^r (control vector)	This study
***Bj* pJNK1**	*B. japonicum* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	This study
***Bj* pJNK5**	*B. japonicum* pUCPM18, Gm^r (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus.*	This study

***Ml* pUCPM18**	*M. loti* with pUCPM18, Gm^r (control vector)	This study
***Ml* pJNK1**	*M. loti* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	This study
***Ml* pJNK5**	*M. loti* with pUCPM18, Gm^r (20μg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	This study
***Sf* pUCPM18**	*S. fredii* with pUCPM18, Gm^r (control vector)	This study
***Sf* pJNK1**	*S. fredii* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	This study
***Sf* pJNK5**	*S. fredii with* pUCPM18, Gm^r (20μg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	This study

Table 3.1: List of bacterial strains used. Detailed characteristics of these strains are given in Table **2.1.** Parent strains and the transformants of *E. coli* and *Rhizobium* were respectively grown at 37°C and 30°C with variations in kanamycin, genatmycin and erythromycin concentrations for rich and minimal media as described in Section **2.2.**

3.2.2 : Development of *B. japonicum* , *M. loti* and *S. fredii* strains harboring *E. herbicola pqq E* gene (pJNK1) and *A. calcoaceticus pqq* gene cluster (pJNK5)

The recombinant plasmids pUCPM18 Gm (control), pJNK1 and pJNK5 were transformed in *B. japonicum, M. loti* and *S. fredii* by electroporation (**Section 2.4.2.2**). The transformants were selected on gentamycin selection plates and were confirmed by RE digestion pattern.

3.2.3: Growth and MPS phenotype of transformant strains of *Rhizobium*

The MPS ability of *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* and its transformants were monitored on Pikovaskya's (PVK) agar and 100 mM

Tris buffered RP (TRP) agar as described in **Section 2.4.**

3.2.4: Effect of heterologous *E. herbicola pqq E* gene (pJNK1) and *A. calcoaceticus pqq* gene cluster (pJNK5) overexpression on the physiology and glucose metabolism.

B. japonicum USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 transformants were subjected to physiological experiments involving growth and organic acid production profiles on TRP medium with 50 mM glucose as carbon source (**Section 2.5**). The samples withdrawn at regular interval were analyzed for O.D.600nm, pH, extracellular glucose and organic acid (**Section 2.8**). The physiological parameters were calculated as in the enzyme assays were performed as described in **Section 2.9.**

3.3: Results:

3.3.1: Heterologous overexpression of *E. herbicola pqq E* gene (pJNK1) and *A. calcoaceticus pqq* gene cluster (pJNK5) in *Rhizobium* strains.

The plasmids incorporated *in B. japonicum*, *M. loti and S. fredii* NGR 234 transformants were isolated from the transformants and were confirmed based on restriction endonuclease digestion pattern (**Fig. 3.1 and 3.2).**

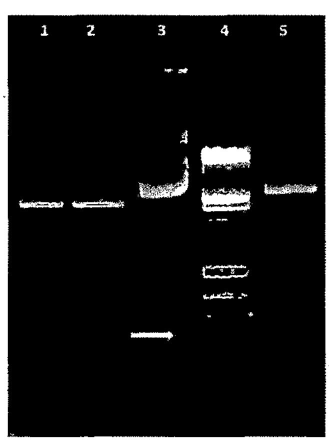

Fig. 3.1: Restriction endonuclease digestion pattern for *Rhizobium* transformants containing pJNK1: Lane 1 and 2 *Bj* pJNK1 and *Ml* pJNK1 BamH1

digested (5.1kb and 1.3 kb), Lane 3. pJNK1 undigested(6.4 kb), Lane 4. EcoR1/Hind III Marker and Lane 5. pJNK1 (6.4 kb) digested with EcoR1.

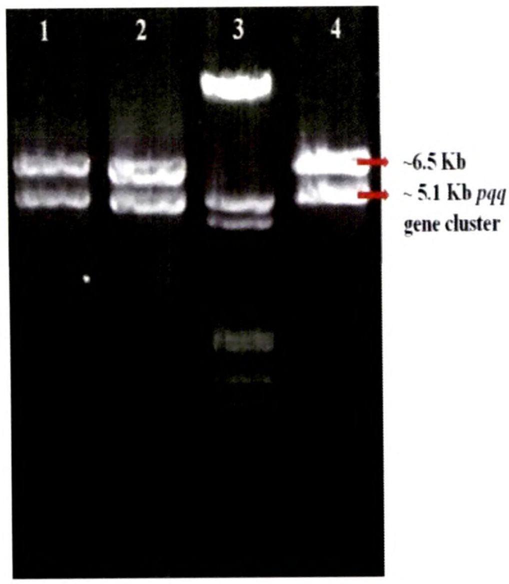

Fig. 3.2: Restriction endonuclease digestion pattern for *Rhizobium* transformants containing pJNK5 (Lane 1, 2 and 4 *Bj* pJNK5, *Ml* pJNK5 and *Sf* pJNK5 EcoR1- BamH1digested (6.5 kb and 5.1 kb), Lane 3- EcoR1/Hind III Marker)

3.3.2 Effect of overexpression of *E. herbicola pqqE* and *A. calcoaceticus pqq* gene cluster (pJNK5) on GDH activity in *B. japonicum, M. loti and S. fredii* NGR 234.

GDH activity in *Bj* (pJNK1) and *Ml* (pJNK1) was found to be ~18 U and ~19 U, respectively, which is ~1.2 to ~1.6 fold compared to native strain (**Fig. 3.3.A, B** and **C**). On the other hand, *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) had ~177 U, ~ 144 U and ~ 210 U of GDH activities, respectively, which is ~14, ~10 and ~15 fold higher compared to native strains.

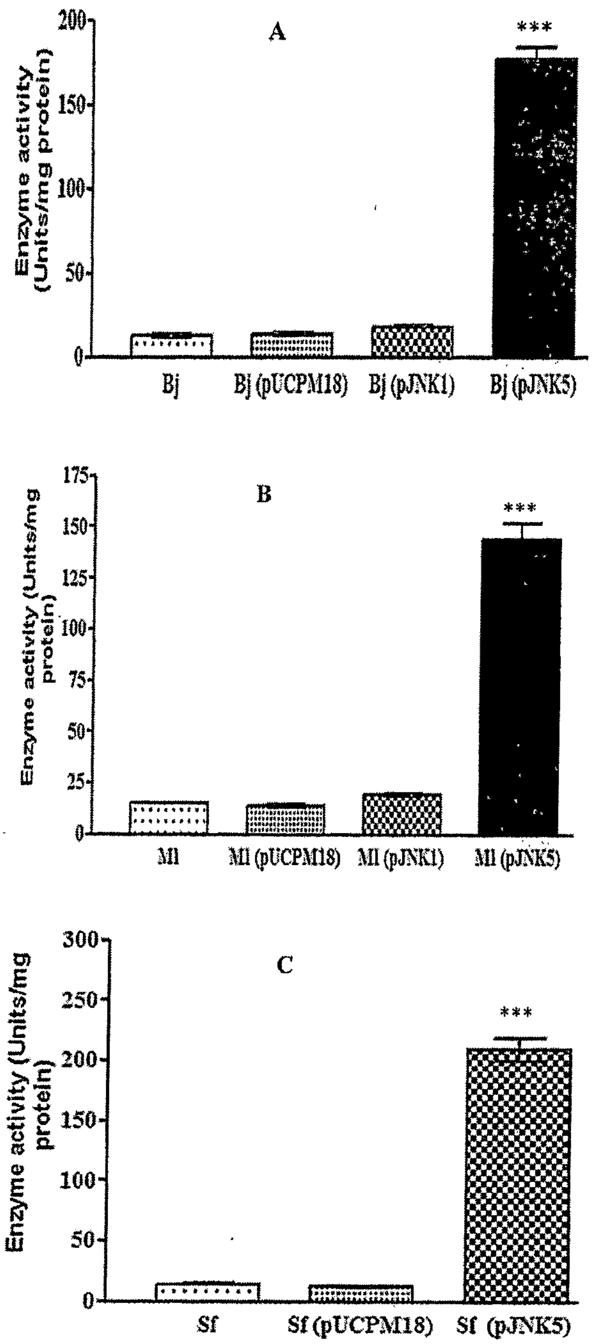

Fig 3.3: GDH activity of *Rhizobium* transformants containing *pqq* gene cluster (A) *B. japonicum*, (B) *M. loti* (C)*S. fredii* NGR 234. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

3.3.3: Growth and MPS ability of *Rhizobium* transformant of *pqq E* and *pqq* cluster *genes.*

Bj (pJNK1) and *Ml* (pJNK1) showed no significant difference compared to native and control both on PVK and TRP plates after 3 days of incubation at 30°C (**Fig. 3.4**). However, *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) transformants showed good phenotype. The pJNK5 transformants of *B. japonicum*, *M. loti* and *S. fredii* showed maximum zone of clearance as compared to the control pUCPM18 and pJNK1 transformants (**Table 3.2).**

where is Bj (pJNK1) fig 3-4

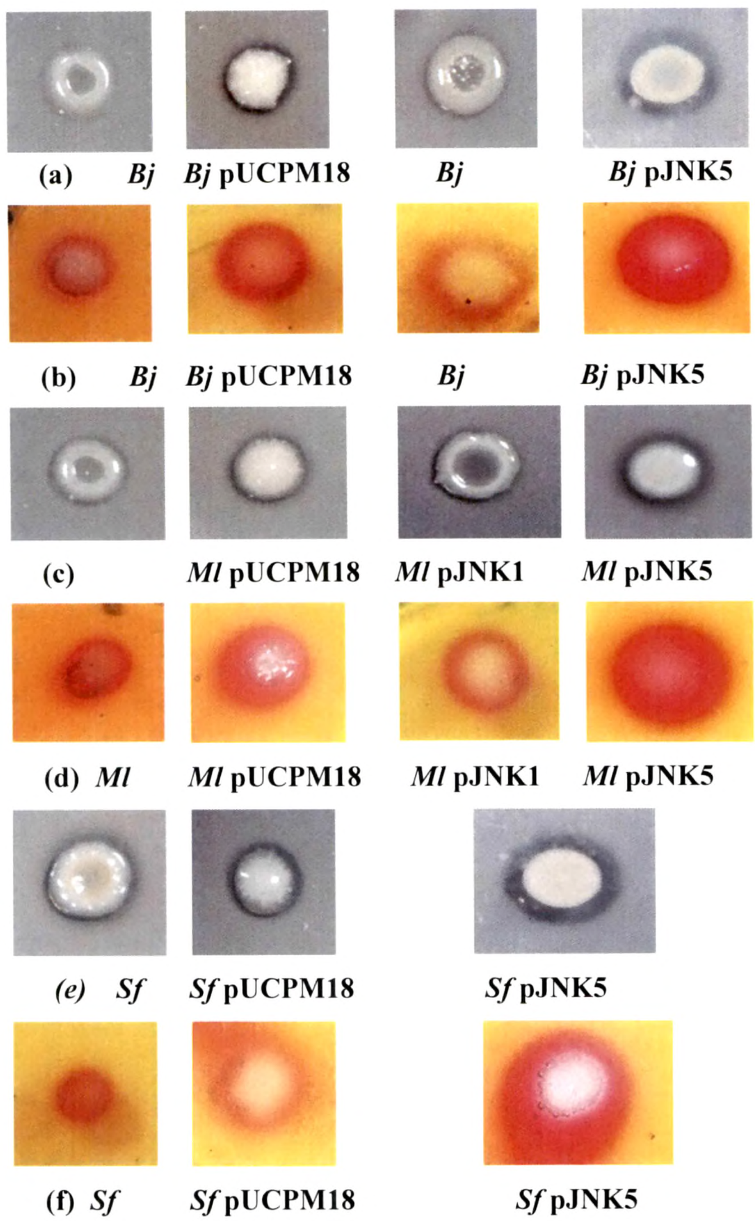

Fig 3.4: MPS phenotype of *B. japonicum, M. loti* and *S.fredii* strains harboring pJNK5 plasmid. (a), (c) and (e) on Pikovskaya's agar and **(b), (d) and (f)** Tris rock phosphate agar containing 50 mM glucose and 100 mM Tris HCl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 °C. Media composition and other experimental details are as described in **Sections 2.4.**

Rhizobium Strains	Diameter of zone of clearance (mm)	Diameter of colony (mm)	Phosphate Solubilizing Index
Bj	12.17 ± 0.29	11.17 ± 0.29	1.09
Bj pUCPM18	11.17 ± 0.29	9.50 ± 0.50	1.22
Bj pJNK1	11.17 ± 0.29	10.50± 0.50	1.06
Bj pJNK5	14.50 ± 0.50	10.17 ± 0.29	1.44
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
Ml pUCPM18	12.17 ± 0.29	10.17 ± 0.29	1.22
Ml pJNK1	12.83 ± 0.29	11.17 ± 0.29	1.14
Ml pJNK5	12.50 ± 0.50	9.17 ± 0.29	1.36
Sf	12.17 ± 0.29	10.50 ± 0.50	1.20
Sf pUCPM18	12.17 ± 0.29	10.17 ± 0.29	1.22
Sf pJNK5	14.50 ± 0.50	11.17 ± 0.29	1.29

What is P solubilization index? Has it been stated anywhere in Materials and methods?

Check this data again

Table 3.2: P solubilization index on Pikovskyas agar of *B. japonicum*, *M. loti and S. fredii* transformants. *Bj, Ml and Sf*: wild type strain; *Bj* p18, Ml p18 and Sf p18 : *B. japonicum*, *M. loti and S. fredii* with vector control and *Bj* (pJNK1 and pJNK5), *Ml* (pJNK1 and pJNK5) and *Sf* (pJNK1 and pJNK5) : *B. japonicum* with *pqq E* and *pqq* gene cluster, *M. loti* with *pqq E* and *pqq* gene cluster and *Sf* with *pqq E and pqq* gene cluster. The results were noted after an incubation of 3 days at 30ºC and are given as mean ± S.D. of three independent observations as compared to native *Bj, Ml* and *Sf.*

B. japonicum is slow growing, M. loti is intermediate and S. fredii is fast growing. Why observations were taken only at 3 days for all strains uniformly.

3.3.3: Effect of *E. herbicola pqq E* gene (pJNK1) and *A. calcoaceticus pqq* gene cluster (pJNK5) overexpression on growth pattern and pH profile in presence of 50mM glucose concentration.

Growth profile and pH drop of Native, control and pJNK1 transformants of *B. japonicum* and *M. loti* on 50 mM glucose in TRP medium showed no significant growth and pH change between them. But pJNK5 transformants of *B. japonicum, M. loti* and *S. fredii* showed maximum growth and pH drop of 4.2, 4.1 and 4.34 respectively within 16 h compared to around pH 6.5 to 6.7 of native and control at 20 h **(Fig 3.5 and Fig 3.6).**

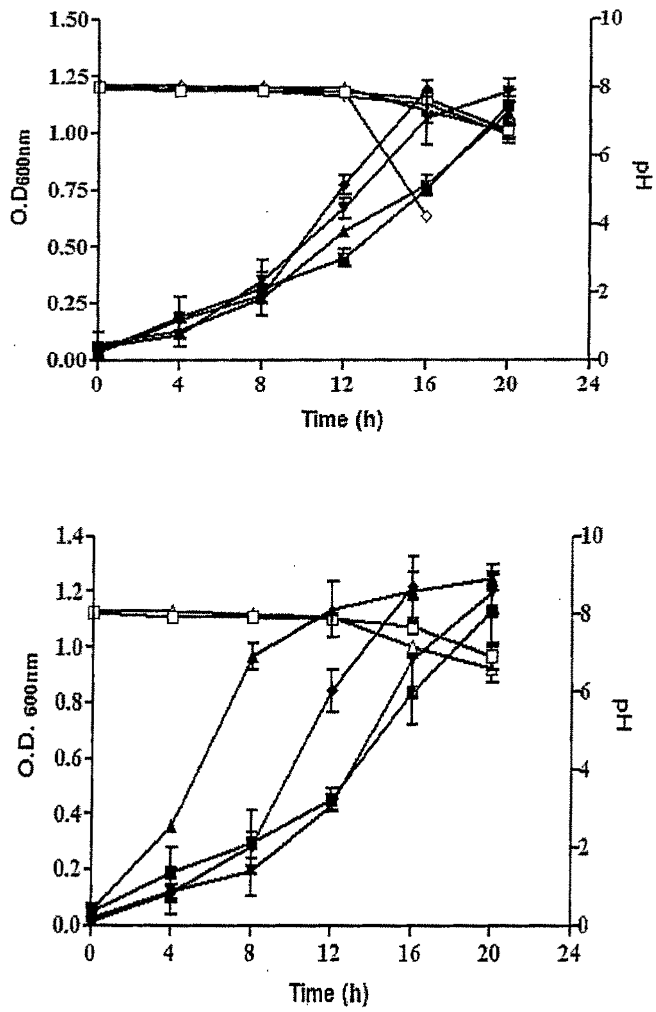

Fig. 3.5: Effect of *pqq* gene cluster overexpression on extracellular pH (□, Δ, ▽,◊) and growth profile (■, ▲, ▼,◆) of (A) *B. japonicum* and (B) *M. loti*, on TRP medium with 50 mM glucose .(□, ■, *Bj, Ml* wild type); {Δ, ▲, *Bj* (pUCPM18Gm^r), *Ml* (pUCPM18Gm^r)}; {▽, ▼, *Bj* (pJNK1), *Ml* (pJNK1)}; {◊, ◆, *Bj* (pJNK5), *Ml* (pJNK5)}. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

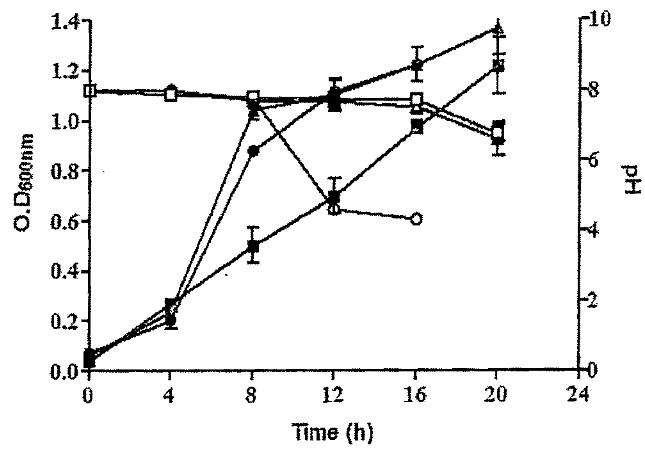

Fig 3.6: Effect of *pqq* gene cluster overexpression on extracellular pH (□, Δ, ○) and growth profile (■, ▲,●) of *S. fredii* on TRP medium with 50 mM glucose .(□, ■, *Sf* wild type); {Δ, ▲, *Sf* (pUCPM18Gm^r), {○, ●, *Sf* (pJNK5). OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

3.3.4: Physiological effects of *A. calcoaceticus pqq* gene cluster overexpression on *B. japonicum*, *M. loti* and *S. fredii* in TRP medium with 50mM glucose.

In presence of 50 mM glucose, increase in GDH activity significantly affected growth profile. The total glucose utilization rate at the time of pH drop remained unaffected but there was ~ 2 fold decrease in glucose consumed due to increase in gluconic acid secretion. However, the Specific Glucose Utilization Rate Q_{Glc} (g.g dcw^{-1}.h^{-1}) decreased by ~1.2 to ~1.9 fold in pJNK5 transformants. Additionally, the increase in GDH activity increased the specific growth rate by ~1.0 to ~2.22 fold, and improved the biomass yield by ~1.0 to ~ 1.6 fold in pJNK5 transformants compared to control (**Table 3.3**).

Rhizobium Strains	**Specific Growth Rate $K(h^{-1})^a$**	**Total Glucose Utilized (mM) ^{b}**	**Glucose Consumed (mM) ^{b}**	**Biomass Yield $Y_{dcw/Glc}$ (g/g) ^{a}**	**Specific Glucose Utilization Rate Q_{Glc} $(g.g\ dcw^{-1}.h^{-1})^a$**
Bj	0.186 ± 0.026	46.20 ± 0.20	38.23 ±1.33	1.78 ± 0.14	0.14 ± 0.01
Bj pUCPM18	0.280 ± 0.051	48.30 ± 0.46	40.65 ± 0.30	1.21 ± 0.16	0.21 ± 0.02
Bj pJNK5	0.413 ± 0.04***	49.20 ± 0.20	18.80 ± 1.43	2.47 ± 0.27***	0.10 ± 0.01
Ml	0.221 ± 0.03	45.91 ± 0.64	37.07 ± 0.55	1.36 ±0.26	0.19 ± 0.04
Ml pUCPM18	0.265 ± 0.02	48.40 ± 0.36	40.38 ± 0.02	1.0 ±0.04	0.26 ± 0.01
Ml pJNK5	0.398 ± 0.06***	49.30 ± 0.31	18.62 ± 3.64	1.61 ± 0.22*	0.16 ± 0.02
Sf	0.260 ± 0.02	46.10 ± 0.42	37.17 ± 0.55	1.69 ± 0.22	0.15 ± 0.02
Sf pUCPM18	0.311 ± 0.02	48.10 ± 0.10	40.67 ± 0.40	1.85 ± 0.10	0.14 ± 0.01
Sf pJNK5	0.543 ± 0.02***	48.07 ± 0.60	17.08 ± 2.49	2.63 ± 0.06**	0.08 ± 0.02

Table 3.3: Physiological variables and metabolic data from of *B. japonicum*, *M. loti* and *S. fredii pqq* transformants grown on TRP medium 100mM Tris-Cl buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml. The results are expressed as Mean ± S.E.M of six independent observations. *a* Biomass yield $Y_{dcw/Glc}$, specific growth rate (k) and specific glucose utilization rate (Q_{Glc}) were determined from mid log phase of each experiment. *b* Total glucose utilized and glucose consumed were determined at the time of pH drop. The difference between total glucose utilized and glucose consumed is as explained. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

3.3.5 : P solubilization and organic acid secretion in 100mM Tris-Cl Buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml.

There was no significant increase in P release by pJNK1 transformants of *B. japonicum* and *M. loti* compared to control and native while ~5, ~9 and ~10 fold increase in P release was seen by pJNK5 transformants of *B. japonicum, M. loti* and *S. fredii* respectively **(Fig. 3.7)**.

On TRP medium in presence of 50mM glucose and 100mM Tris Cl Buffer pH 8.0, the organic acids identified were mainly gluconic and 2-ketogluconic acids. Extracellular medium of pJNK5 transformants of *B. japonicum, M. loti* and *S. fredii* contained ~14, ~10 and ~15 folds higher amounts of gluconic acid, respectively, with its specific yield ($Y_{C/G}$) increasing by ~2.2, ~2.7 and ~1.7 fold. Levels of 2-ketogluconic acid were unaltered as compared to native and controls (**Table.3.4**).

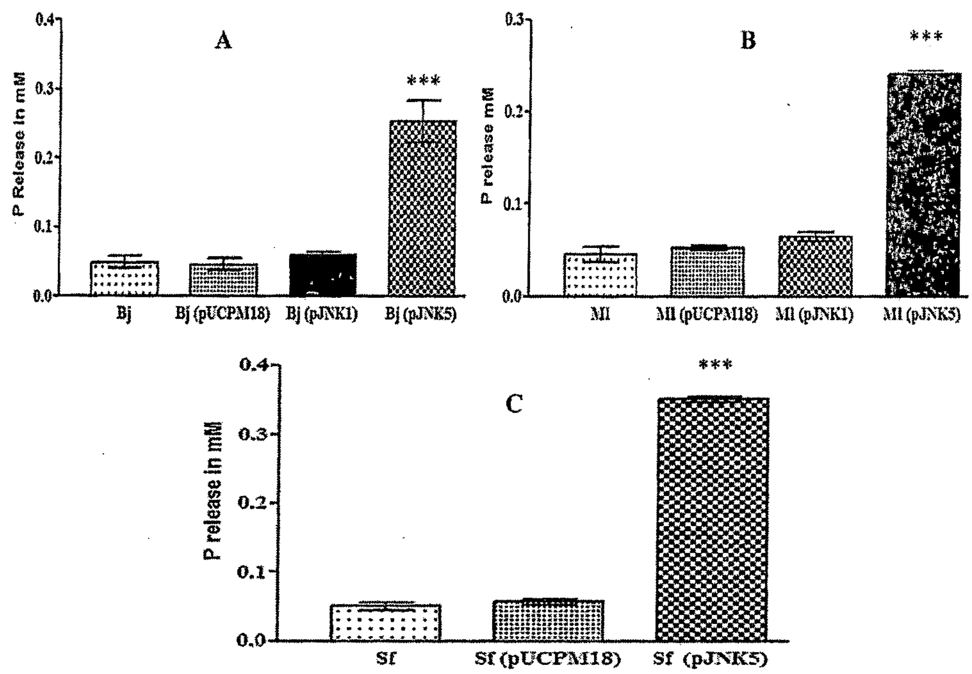

Fig.3.7: Phosphate released by *Rhizobium* transformants containing *pqq* gene cluster (A) *B. japonicum* (B) *M. loti* and (C) *S. fredii*. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

Rhizobium Strain	**GA mM**	**Specific Yield $Y_{C/G}$**
Bj	6.26 ± 0.23	1.39 ± 0.12
***Bj* pUCPM18**	5.72 ± 0.06	1.39 ± 0.08
***Bj* pJNK5**	28.33 ± 0.88***	3.11 ± 0.15***
Ml	6.533 ± 0.18	1.01 ± 0.21
***Ml* pUCPM18**	5.80 ± 0.10	0.98 ± 0.03
***Ml* pJNK5**	28.43 ± 2.14***	2.75 ± 0.19***
Sf	6.760 ± 0.10	1.59 ± 0.05
***Sf* pUCPM18**	5.50 ± 0.30	1.10 ± 0.11
***Sf* pJNK5**	29.00 ± 1.73***	2.77 ± 0.27***

Table 3.4: Organic acid Secretion and Organic acid yield from of *B. japonicum*, *M. loti* and *S. fredii pqq* transformants grown on TRP medium 100mM Tris-Cl buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

3.3.6: Effect of *E. herbicola pqq E* gene (pJNK1) and *A. calcoaceticus pqq* gene cluster (pJNK5) overexpression on PQQ secretion in *Rhizobium* transformants.

Bj (pJNK1) and *Ml* (pJNK1) secreted ~0.315 μM and ~0.159 μM PQQ in medium, respectively, while *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) secreted ~7.30 μM, ~7.00 μM and ~8.75 μM PQQ **(Fig. 3.8)** lot of variation is found in PQQ secretion in bacteria **(Table 3.5)**. There was no significant increase in PQQ secretion of *Bj* (pJNK1) and *Ml* (pJNK1) compared to native and control, while in *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) ~ 28, ~70 and ~ 40 fold increase in PQQ secretion was found.

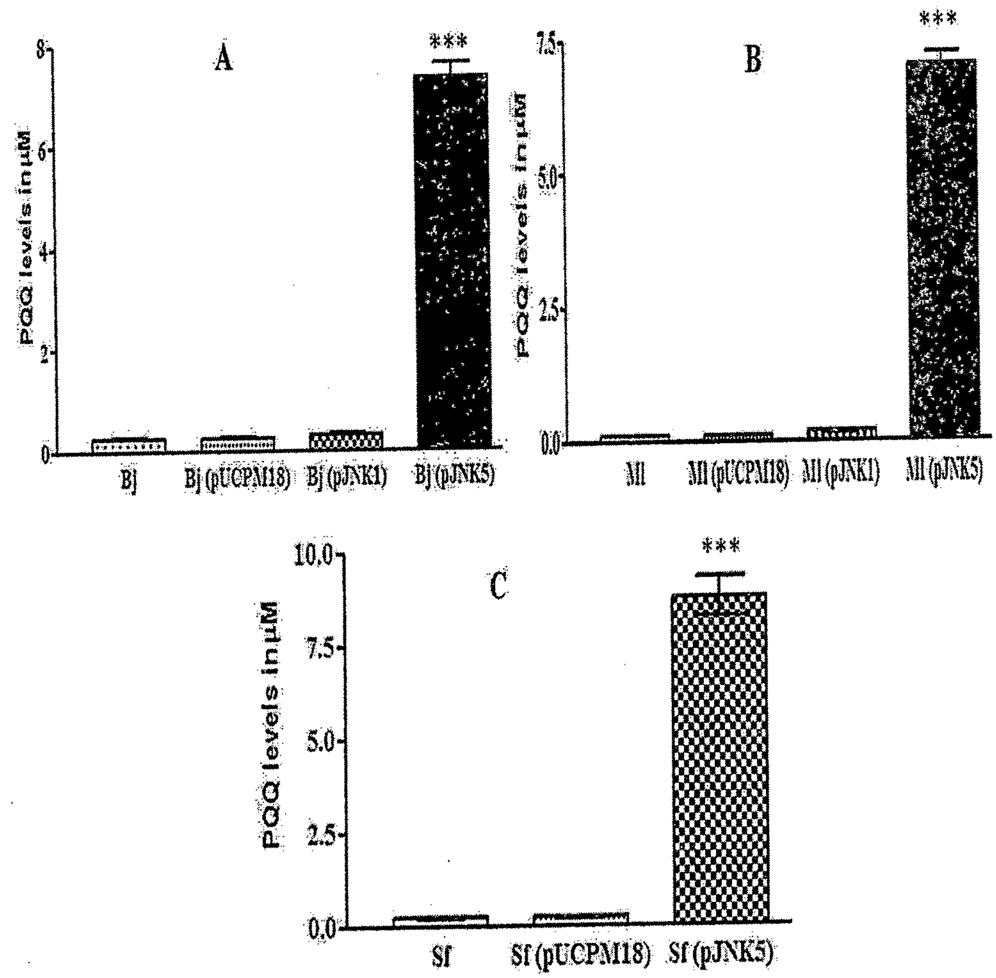

Fig. 3.8: PQQ secreted by *Rhizobium* transformants containing *pqq* gene cluster (A) *B. japonicum* (B) *M. loti* and (C) *S. fredii.* The results are expressed as Mean ± S.E.M of six independent observations * P<0.05, ** P<0.01 and *** P<0.001.

Table 3.5: Variation of PQQ secretion in bacteria.

Host	Nature of the genes incorporated	Amount of PQQ in the medium	Ref.
E. coli K12	*A. calcoaceticus pqq* gene cluster	Very Low	Goosen et al., 1989
E. coli	*Klebsiella pneumoniae* pqq gene cluster	0.18 µM	Meulenberg et al., 1995
E. coli (JM109, BL21)	*G. oxydans* M5 *pqqABCDE* gene cluster	6 µM*	Yang et al., 2010
Methylovorus sp. MP688		45 µM*	Ge et al., 2013
P. aeruginosa ATCC 17933	-	0.455 µM*	Gliese et al., 2010
H. seropedicae Z67	*Erwinia herbicola pqqE*	0.011 ± 0.002 µM	Wagh 2013
H. seropedicae Z67	*Acinetobacter calcoaceticus pqq* gene cluster	1.10 ± 0.64 µM	Wagh 2013
H. seropedicae Z67	*Pseudomonas fluorescens* B161 *pqq* gene cluster	2.55 ± 0.10 µM	Wagh 2013
E. asburiae PSI3	-	4.52 ± 0.84 µM	Wagh 2013
P. aeruginosa P4	-	1.41 ± 0.12 µM	Wagh 2013

3.3.7: Effect of *A. calcoaceticus pqq* gene cluster (pJNK5) overexpression on Biofilm, EPS and IAA secretion in *Rhizobium* transformants.

Biofilm and exopolysaccharide synthesis on an average showed significant increase by ~2.5 fold, while ~ 1.8 fold increase in IAA secretion in *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) transformants in 100 mM Tris-Cl buffer pH 8.0, 50 mM glucose and rock phosphate 1 mg/ml in comparison to native and control (**Table 3.5**).

***Rhizobium* Strains**	**Biofilm O.D.at 550nm**	**EPS (g/100ml)**	**IAA (µg/ml)**
Bj	1.39 ± 0.02	11.48 ± 0.1	20.14 ± 1.33
***Bj* pUCPM18**	1.45 ± 0.03	12.56 ± 0.2	22.32 ± 1.65
***Bj* pJNK5**	3.89 ± 0.04***	24.54 ± 0.3***	39.53 ± 1.86***
Ml	1.51 ± 0.06	11.34 ± 0.05	28.17 ± 1.35
***Ml* pUCPM18**	1.81 ± 0.05	11.89 ± 0.1	29.67 ± 1.81
***Ml* pJNK5**	4.21 ± 0.04***	28.33 ± 0.5***	42.16 ± 2.00***
Sf	1.61 ± 0.10	12.53 ± 1.54	32.79 ± 1.87
***Sf* pUCPM18**	2.10 ± 0.16	13.43 ± 1.11	34.10 ± 1.32
***Sf* pJNK5**	4.52 ± 0.2***	30.31 ± 1.88***	52.12 ± 1.22***

Table 3.6: Biofilm, Exopolysaccharide and Indole acetic acid production by *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) transformants in TRP medium The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

Discussion

Gram Negative bacteria like *E. coli*, *Azospirillum*, *Herbaspirillum* show presence of apoGDH and lack *pqq* genes ; while *Acinetobacter, Gluconobacter, Pseudomonas, Erwinia* species possess both *pqq* and *gcd* genes. Addition of exogenous PQQ, or incorporation of *pqq* gene/genes cluster reconstitutes apoGDH to active form in many Gram negative bacteria like *E. coli*, *R. leguminosarum*, *A. lwoffi*, *Pseudomonas sp.*, *Burkholderia*, *Azospirilluim brasileinse* (Van Schie et al., 1986; Goldstein and Liu, 1987; Liu et al. 1992; Vikram et al., 2007).

Diverse numbers of genes are found in bacteria for PQQ synthesis (Goldstein and Liu, 1987; Goosen et al., 1992; Liu et al. 1992; Vikram et al., 2007; Choi et al., 2008; Shen et al., 2012). Genome of *Rhizobium* species such as *B. japonicum*, *S. fredii*, *S. meliloti* encode for *pqq* genes, whereas it is absent in *M. loti* and *R. leguminosarum*. Overexpression of *E. herbicola pqqE* and *R. aquatilis pqqED* in *E. coli* HB101 conferred MPS ability, whereas in our studies overexpression of *E. herbicola pqq*E (pJNK1) in apoGDH harboring *B. japonicum* and *M. loti* did not show significant release of GA and PQQ secretion compared to native and control. Similar effects are seen in overexpression of *pqqE* gene in *H. seropedicaea* Z67. A reason for the variations upon *pqqE* overexpression in different bacteria is not clear (Wagh, 2013).

B. japonicum, *M. loti* and *S. fredii* NGR234 transformant containing pJNK5 with *A. calcoaceticus pqq* genes in pUCPM18Gm^r under P*lac* promoter showed increase in GDH activity by ~ 13 fold on M9 medium. This demonstrates that apoGDH present in *Rhizobium* strains is functional and constitutive P*lac* is a strong promoter giving significant overexpression. Similar results were found with *Hs* Z67(pJNK5) which showed 221 U of GDH activity while control plasmid transformant had no detectable activity (Wagh, 2013) .

Overexpression of *pqq* gene clusters of *A. lwoffi* , *D. radiodurans*, *E. intermedium* and *S. marcescens* in *E. coli* showed GA secretion and MPS ability (Krishnaraj and Goldstein 2001; Apte et al., 2003; Kim et al., 2003). Overexpression of *A. calcoaceticus pqq* gene cluster (pJNK5) in *B. japonicum* USDA110, *M. loti* MAFF030669 and *S. fredii* NGR234 led to secretion of ~ 25 mM GA and solubilized ~0.27 mM P on 100 mM Tris-Cl pH 8.0 buffered rock phosphate medium containing

50 mM glucose. Similar results were found with transformant *Hs* (pJNK5) which secreted 23.47 mM GA and solubilized 0.24 mM P on 100 mM HEPES RP medium containing 50 mM glucose.

All *Rhizobium* transformants secreted ~7.5 µM of PQQ, which is significantly higher than *Hs* (pJNK5) transformant which secreted 1.10 µM, natural phosphate solubilizing rhizobacteria *E. asburiae* PSI3 and *P. aeruginosa* secreted 4.5 µM and 1.4 µM PQQ, respectively (Wagh, 2013). 1 µM of synthetic PQQ shows 25 % increase in fresh weight of cucumber, increase in fresh and dry weights of *Arabidopsis* and the size of the cotyledons of hot pepper treated with 25 nM PQQ was seen (Choi et al., 2008). Increase in root and shoot weight of mung plant by 25 % was observed between wild type and *pqq* mutant CMG860 (Ahmed and Shahab, 2010). It would be interesting to check plant growth promoting activity of PQQ producing genetically modified Rhizobium strains.

S. meliloti GDH mutant showed impaired symbiotic phenotype and inefficient nodulation ability (Bernardelli et al., 2008). Our study shows apoGDH of *Rhizobium* strains reconstituted into active form by overexpression of *A. calcoaceticus pqq* gene cluster (pJNK5) in *B. japonicum*, *M. loti* and *S. fredii* NGR234. Functional GDH of *Rhizobium* strains along with PQQ will play signifcant role in Nitrogen fixation and plant growth.

Overexpression of *A. calcoaceticus pqq* gene cluster in *B. japonicum*, *M. loti* and *S. fredii* NGR234 enhanced EPS production. Phosphate plays as a positive regulator of EPS production, EPS production increased when supplemented with 0.1-20 mM P (Janczarek et al., 2011). *S. meliloti* produces two types of EPS, and the concentration of phosphate in the medium regulates the production of one type of EPS at the expense of the other. Under low-phosphate conditions EPS II predominates, and the colonies of these bacteria have a more mucoid morphology. Under normal conditions, *S. meliloti* produces EPS I, with less mucoid colonies (Zhan et al., 1991; Mendrygal & Gonzalez, 2000). EPS has been shown to play an essential role for the effective establishment of the symbiosis between *Rhizobium* sp. NGR234 and *L. leucocephala* or *M. atropurpureum* (Bomfeti et al., 2011). EPS mutant strains did not show nodule formation with *Glycyrrhiza uralensis* (Wang et al., 2008). The strains with high levels of EPS production tend to be more tolerant to acidic conditions and salinity than strains that produce low EPS levels (Cunningham and Munns, 1984; Eaglesham et al., 1987; Xavier et al., 1998; Freitas et al., 2007; Xavier et al., 2007). Thus ~2.5 fold increase in EPS production will help in enhancing the movement of rhizobium infection thread and symbiosome formation. EPS along with LPS and BacA increase the competitiveness of *Rhizobium* nodulation. PGPR function of *Rhizobium* transformants.

Correct the sentence.

Overexpression of *A. calcoaceticus pqq* gene cluster in *B. japonicum*, *M. loti* and *S. fredii* NGR234 enhanced Biofilm formation. Biofilm formation in *S. meliloti* is altered by changes in environmental conditions and the nutritional status of the medium (Rinaudi et al., 2006). Biofilm formation by *Pseudomonas aureofaciens* PA147-2 requires a threshold concentration of extracellular inorganic phosphate (Monds et al., 2001). Phosphate starvation in *P. aureofaciens* PA147-2 shows a *pho*B-dependent decrease in biofilm while, phosphorus limitation and *pho*B overexpression increase the density of *A. tumefaciens* biofilms (Danhorn and Fuqua, 2007).

EPS I and II of *S. meliloti* strain nodulating alfalfa showed a correlation between EPS and biofilm production for cell-cell interactions and surface attachment (Sorroche et al., 2012). Many species of beneficial soil bacteria, including rhizobia, form microcolonies or biofilms when they colonize roots (Bogino et al., 2013). The production of EPSs on plant surfaces or tissues allows bacterial colonization and biofilm formation (Rinaudi et al., 2009). The beneficial roles of bacteria on plants are related to these abilities. *S. meliloti* Exo-(*exo*Y) mutants elicit on alfalfa roots ineffective (Fix^-), meristemless nodules that are not colonized internally by rhizobia and biofilming ability of the *exo*Y strain Rm7210 was ~ 57–60% reduced compared to wild-type Rm1021 strain (Fujishige et al., 2006). Our study shows ~2.5 fold increase in EPS as well as Biofilm formation along with P release; this will give a stimulatory effect to transformed *Rhizobium* strains for root colonization and MPS ability.

Direct promotion of growth occurs when PGPR provide compounds that affect plant metabolism. Besides biological nitrogen fixation, the most important direct

PGPR mechanism is synthesis of phytohormones or plant growth regulating compounds. Examples are production of indole- 3-acetic acid (IAA) by *A. diazotrophicus*, *H. seropedicae*, zeatin and ethylene by *Azospirillum* spp., gibberellic acid (GA3) by *A. lipoferum* strain op33 and abscisic acid (ABA) by *A. brasilense* strains Cd and Az39 (Boiero et al., 2007). The ability to synthesize phytohormone is widely distributed among plant associated bacteria. 80% of the bacteria isolated from plant rhizosphere are able to produce IAA. IAA play a role in one or more aspects of nodule growth and development and it is detected in increased levels in nodule tissue (Sahasrabudhe, 2011). IAA enhances root proliferation in *A. brasilense* and *P. putida* GR12-2 (Faure et al., 2009). Inoculated biofilm of *Penicillium* spp.–*Bradyrhizobium* spp. showed increased IAA release which increased root growth of soybean (*Glycine max*) (Jayasinghearachchi and Seneviratne, 2004a). *S. meliloti* strain overproducing IAA showed enhanced P solubilization (Bianco et al., 2010).

Our studies show that, with increased release of P in TRP medium, EPS and Biofilm increased by 2.5 fold compared to native and control, IAA secretion increased by ~ 2 fold. This shows that overexpression of *A. calcoaceticus pqq* gene enhanced MPS ability of *B. japonicum*, *M. loti* and *S. fredii* NGR234, and augments its role as PGPR.