Evaluating the effect of engineered genetic modifications on P-solubilization and plant growth promotion ability of fluorescent pseudomonads

CHAPTER 6

6 Chapter

6.1 INTRODUCTION

Phosphorus (P) is one of the major essential macronutrients limiting the plant growth and is applied to the soil as chemical phosphate fertilizer. Of the total soil P, only 1 to 5% is in a soluble and plant-available form. The overall P use efficiency following phosphate fertilizer application is low because of the rapid formation of insoluble complexes (Goldstein and Krishnaraj, 2007; Vassilev and Vassileva, 2003). Hence, during agricultural crop production frequent application of soluble forms of inorganic P cause leaching to the ground water and results in eutrophication of aquatic systems (Del Campillo et al., 1999; Kloepper 2009). To maintain the sustainability and soil fertility research efforts are concentrated to make use of less expensive, eco friendly sources of P nutrients such as rock phosphate (Whitelaw, 2000; Arcand 2006). Rock phosphate originates from igneous, sedimentary, metamorphic, and biogenic sources, with sedimentary being the most widespread (van Straaten, 2002). Microorganisms are an integral part of the soil P cycle and in particular, are effective in releasing P from rock phosphate (Richardson 2011). Therefore, phosphate solubilizing microorganisms with other plant growth promoting abilities have attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield (Goldstein et al., 1999; Armarger, 2002; Fasim et al., 2002; Khan et al., 2007).

Plant growth promoting rhizobacteria (PGPR) are soil bacteria that can benefit plant growth by different mechanisms (Glick, 1995; Archana et al., 2012), and P-solubilization ability of the microorganisms is considered to be one of the most important traits. The use of PGPR as phosphate biofertilizer is advantageous in the sustainable agricultural practices. The mechanism of mineral phosphate solubilization by PGPR strains is associated with the release of low molecular weight organic acids mainly gluconic citric and oxalic acid which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Goldstein, 1995; Chen et al., 2006). However, P-solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes et al., 1999).

PGPR benefit the plant growth directly by solubilization of insoluble phosphorous, nitrogen fixation, sequestering of iron by production of siderophores, producing metabolites such as auxins, cytokinins, gibberellins, lowering of ethylene concentration. The indirect growth promotion occurs via antibiotic production, synthesis of antifungal metabolites and cell wall lysing enzymes, competing for sites of root colonization, induced systemic resistance (Ahemad and Khan, 2011). The premier example of PGPR agents occur in many genera including *Actinoplanes, Agrobacterium, Alcaligens, Amorphosporangium, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Cellulomonas, Enterobacter, Erwinia, Flavobacterium, Gluconacetobacter, Microbacterium, Micromonospora, Pseudomonas, Rhizobia, Serratia, Streptomyces, Xanthomonas*, etc. One of the dominant genera among PGPR is *Pseudomonas* spp. *Pseudomonas sp.* is widespread bacteria in agricultural soils and has many traits that make them well-matched as PGPR; the most effective strains have been fluorescent *Pseudomonas* spp. The selective advantages of fluorescent pseudomonads as PGPR includes: (i) grow rapidly and utilize seed and root exudates; (ii) colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant; (iii) produce a wide spectrum of bioactive metabolites (i.e., HCN, antimicrobials, siderophores, volatiles, proteases, phosphate solubilizing enzymes and growth promoting substances) and are responsible for the natural suppressiveness of some soils to soil borne pathogens; (iv) compete aggressively with other microorganisms; and (v) adapt to environmental stresses (Lugtenberg 2009; Aeron et al., 2011, Saharan et al., 2011; Akhtar et al., 2012).

The beneficial effects of PGPR seen under greenhouse conditions are often not repeatable under field conditions and the results in terms of crop growth and yields are highly variable (Gyaneshwar et al. 2002; Viveros –Martinez et al., 2010). Understanding the influence of environmental factors is widely recognized as a key to improve the level and reliability of PGPR (Dutta et al., 2010). Over-expression of genes involved in soil inorganic phosphate solubilization in natural PGPR can improve the capacity of microorganisms when used as inoculants (Bashan et al., 2000). Studies carried out so far have shown that following appropriate regulations, genetically modified microorganisms can be applied

safely in agriculture (Armarger, 2002; Morrissey et al., 2002). Chromosomal insertion of the genes is one of the tools to minimize the risks of using genetically modified microbes in agricultural filed.

The dissemination of bacteria in the field has remained marginal, with the exception of rhizobia and agrobacteria. Inoculation of plants with P-solubilizing microorganisms in controlled experiments improved the growth and P nutrition, especially under glasshouse conditions and in fewer cases in the field (Whitelaw, 2000; Gyaneshwar *et al.*, 2002; Jakobsen *et al.*, 2005; Khan *et al.*, 2007; Rodríguez and Fraga, 2006; Harvey *et al.*, 2009; Khan *et al.*, 2010). But, the effectiveness of PSMs in the laboratory or controlled conditions may not be operable in soils (Richardson 2011). Plant growth promotion abilities of biofertilizers are strongly influenced by climate changes and are restrictive to certain cultivars, climate, and soil conditions (Figueiredo et al., 2010; Kern et al.2011). The phosphate solubilization can be accompanied by a number of activities such as production of plant stimulants, enzyme production, biocontrol activity and organic acid production (Vassilev et al. 2006; 2007a; 2007b; 2008; 2009b).

Efficacy of PGPR in field conditions is determined mainly by their survival in harsh environmental conditions including high concentrations of environmental contaminants, salts, extremes of pH and temperature, and competition with other organisms. Isolation of stress tolerant PSM is gaining importance to enhance the efficacy of PSM (Thakuria et al. 2004; Chaiharn et al., 2008; Vassilev 2012). A large number of PGPR representing diverse genera have been isolated over past 50 years. Despite of their natural means of plant growth promotion ability, strains are genetically modified because of their inconsistent performance and requirement of uneconomically high dose application (Carmen, 2011). Additionally, several plant growth promoting traits can be combined in a single organism for long-term cell survival under a variety of environmental conditions (Defez, 2006). Inoculation of *Medicago tranculata* plant with indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti* improved both the shoot and root fresh weigh, nitrogen fixation ability, P mobilization, oxidative damage and salt tolerance (Imperlini et al., 2009; Bianco and Defez,

2009; 2010a,b). Hence, genetic modification of native strains may help to survive, adapt and function better in the rhizosphere and improve plant nutrition.

Catabolic versatility of the fluorescent pseudomonads appears to be orchestrated by specific regulatory network of central metabolism which is exemplified by the diversity in the nature and number of enzymes of the *anaplerotic node*, their allosteric regulation and gene expression (Sauer and Eikmanns, 2005). Genetic engineering strategies developed for a particular organism may not necessarily work for other organism or even organism of the same species. Therefore, it is necessary to investigate the effects of genetic modification in multiple host organisms. In this study, six diverse *P. fluorescens* strains were subjected to similar genetic modification for citric acid secretion leading to P solubilization and plant growth promotion.

6.2 WORK PLAN

6.2.1 Bacterial strains

Fluorescent pseudomonad strains used in the study are lised in the table 6.1

P. fluorescens **strains/Plasmids**	**Characteristics**	**Source/Reference**
PfO-1	6.4Mb, 60.5%GC, 5736genes, (Accession CP000094)Wild type strain	A generous gift of Prof. Mark Sylvi, USA (Silby, 2009)
Pf-5	Cotton rhizopsheric isolate produces DAPG, Plt, and Prn, ACC(+), L-Methionine (+) 7Mb, 63.3%GC, 6137 genes, (Accession CP000076)	A generous gift of Prof. L. Thomashow USA
CHAO-1	Tobacco rhizopsheric isolate, naturally suppressive to black root rot caused by *Thielaviopsis basicola* , produces DAPG, Plt, Prn, HCN, IAA,ITS, Phz, salicylic acid, pyochelin, a pyoverdine siderophore	Weller, 2007

	(pseudobactin), and other bioactive metabolites. ACC(-), L-Methionine (+).	
ATCC13525	Wild type strain	ATCC
P109	Wild type strain, ACC(-), P^+	A generous gift of Prof. B. N. Johri
Fp315	Wild type strain, ACC(-), P^+	A generous gift of Prof. B. N. Johri
Pf(pYC)	*P. fluorescens* strains with pYC; Gm^r	Ch4
Pf (Int)	*P. fluorescens* strains genomic integrant, Gm^r	Ch5

Table 6.1 : List of plasmids and bacterial strains used in the study. DAPG: 2, 4–Diacetyl pholorglucinol, HCN: Hydrogen Cyanide, IAA: Indole-3-acetic acid, ITS: internal transcribed spacer, Phz: Phenazine, Pit: Pyoluteorin, Prn: Pyrrolnitrin,

All the strains were grown in shake flask using TRP (pH 8.2) medium containing 75mm Tris HCl and 75 mM glucose. For inoculums preparation during plant experiment the cultivation was carried out at 30 °C in an orbital shaker at 200 rpm until all the culture reached to an OD of 0.8-1.0.

6.2.2 In vitro characterization of genomic integrants for P- solubilization ability

The di-calcium phosphate solubilizing ability of the wild type and the transformant strains of fluorescent pseudomonads was tested on Pikovskaya's agar (Pikovskaya, 1948). Bacterial inoculums were prepared by aseptically harvesting the Luria-Bertanni broth grown cultures after washing the cell-pellet twice with 0.85% saline and re-suspending in 1 ml of the same under sterile conditions. Resultant cell suspension was aseptically spotted on the agar plates and was allowed to dry completely followed by incubation at 30 °C for 5–7 days. Rock phosphate solubilizing ability was monitored using 75 mM Tris buffered TRP minimal agar medium (Buch et al., 2008) following the same procedure mentioned above. P-Solubilization on Pikovskaya's agar was determined by monitoring the phosphate solubilization index (PSI) whereas the growth and formation of a red zone on buffered

Senegal rock phosphate Methyl Red agar plates were used to demonstrate P-solubilization and acid production in TRP agar medium using rock phosphates as the mineral P.

Batch culture studies were performed by shaking 30 ml of the TRP minimal broth medium (in 150 ml conical flasks), inoculated to an initial cell density at 600 nm (OD600) of about 0.1 (approximately 10^8cfu/ml), on a rotary shaker maintained at 30 °C with agitation speed of 200 rpm. Drop in the media pH from pH 8.0 to < 5 on TRP broth was used to indicate rock-phosphate solubilization. Quantitative estimation of phosphate solubilization in broth was carried out in culture supernatant by Aemes method (..). In each case, three replicates were used.

6.2.3 Soil preparation

The soil used for the experiment was collected from the uncultivated land at Amri Village, 3 Km away from Navsari Agricultural University, Navsari Gujarat and analysis of the sample prior to experiments showed 1001.0 Kg/ha, 2.32 Kg/ha, 96.48 Kg/ha NPK respectively with a pH 8.4 (1:2.5) and EC (1:2.5) 0.27. The soil pH was determined in 1:2.5 soil:water suspensions using an automatic glass electrode pH meter (Systronic model-361). Phosphorus was evaluated by phosphomolybdic acid methods (Trivedy et al., 1998).

6.2.4 Seed surface sterilization and seed bacterization

Seeds of *Vigna radiata* GM4 obtained from Navsari Agriculture University, Navsari, Gujarat and were washed repeatedly with autoclaved distilled water and soaked in distilled water for 10 minutes. Later seed directly soaked into respective cultures. For uniform treatment of the seed with culture, flasks were kept in an orbital shaker at 500 rpm for 2 h. The average bacterial counts were 10^8 cfu/seed. Seed were treated with *P. fluorescens* strains each containing wild type strain (WT), plasmid transformants of *yc* operon (pYC) and *yc* operon genomic integrant (Int). In all experiments, two controls were used. One is absolute control where no fertilizer and no inoculum was added designated as untreated (UT) and the other control was single super phosphate (SSP) control where recommended dose of SSP was added as soil application with no added inoculum.

6.2.5 Greenhouse experiment

Bacteria coated Mung bean seeds were shown in polybags containing unsterile field soil and reared in a green house (25-30 °C). The pots were irrigated time to time to maintain the moisture level in green house. The growth parameters were recorded and biochemical characterization was carried out at 45 days after emergence. Each treatment had 5 replications (6 seeds per replicate).

6.2.6 Growth parameter assessment

All the plant growth parameters were estimated at 45 days after sowing (DAS).

6.2.7 Biochemical characterization

All antioxidant enzyme specific activities (SOD, Catalase, Gpox, APX) and Acid Phosphatase and NR Specific activities were monitored

6.2.8 Statistical analysis

The experiments were carried out in a completely randomized design (CRD) for *Vigna radiata*. The experimental data was analyzed statistically at 5% level of significance using two factorial CRD analysis.

6.3 RESULTS

6.3.1 P solubilization phenotype characterization of *P. fluorescens yc* operon genomic integrant strains in buffered and unbuffered media.

Genomic integrants *P. fluorescens* PfO-1 and Fp315 *yc* operon showed maximum di calcium phosphate solubization ability on Pikovskaya's agar as compared to the plasmid transformants of *yc* operon (pYC) and plasmid transformants of only citrate synthase without any external transporter (pYF) and wild type. Phosphate solubilization index when calculated varied in the order of PfO-1WT< PfO-1(pGm) < PfO-1(pYF) < PfO-1(pYC) < PfO-1(Intyc). Fp315 showed similar pattern of P solubilization when tested on both di calcium and buffered rock phosphate medium (**Fig. 6.1**). Based on this phenotype, the *P. fluorescens* strains were characterized based on their solubilization ability both on Pikovskaya's agar and TRP agar medium (**Fig. 6.2-6.3**). All strains showed significant

improvement on solubilization index as compared to their respective wild type control (**Fig. 6.4**).

On TRP medium, all cultures showed an improvement in the zone of colouration as compared to the wild type. Moreover, the genomic integrants of PfO-1, Pf-5 and Fp315 showed faster rock phosphate solubilization as evident from the appearance of zone of coloration within 48-72 h of growth as compared to 96-120 h of plasmid transformants and wild type. Except Fp315 wild type, none of the wild type cultures could show zone of coloration on TRP medium containing 75mM Tris HCl (pH 8.2) and 75 mM glucose.

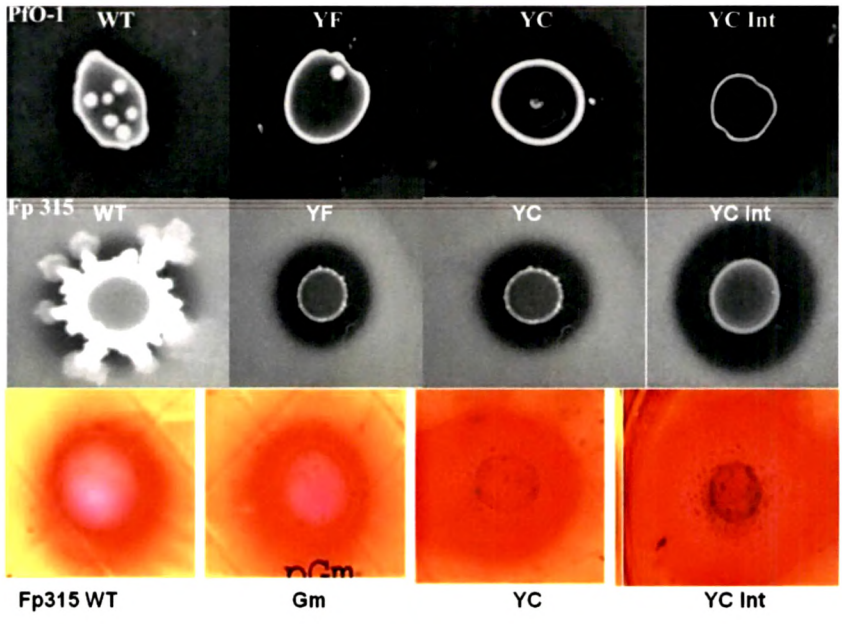


Figure 6.1: P solubilization phenotype of transgenic *P. fluorescens*PfO-1 and Fp315 strains during 96-120 h of growth. WT: wild type strain; Gm: *P. fluorescens with* pUCPM18Gm vector; YF: *P. fluorescens* with pYF plasmid; YC: *P. fluorescens* with pYC plasmid; YCInt: *P. fluorescens* yc operon genomic integrant.

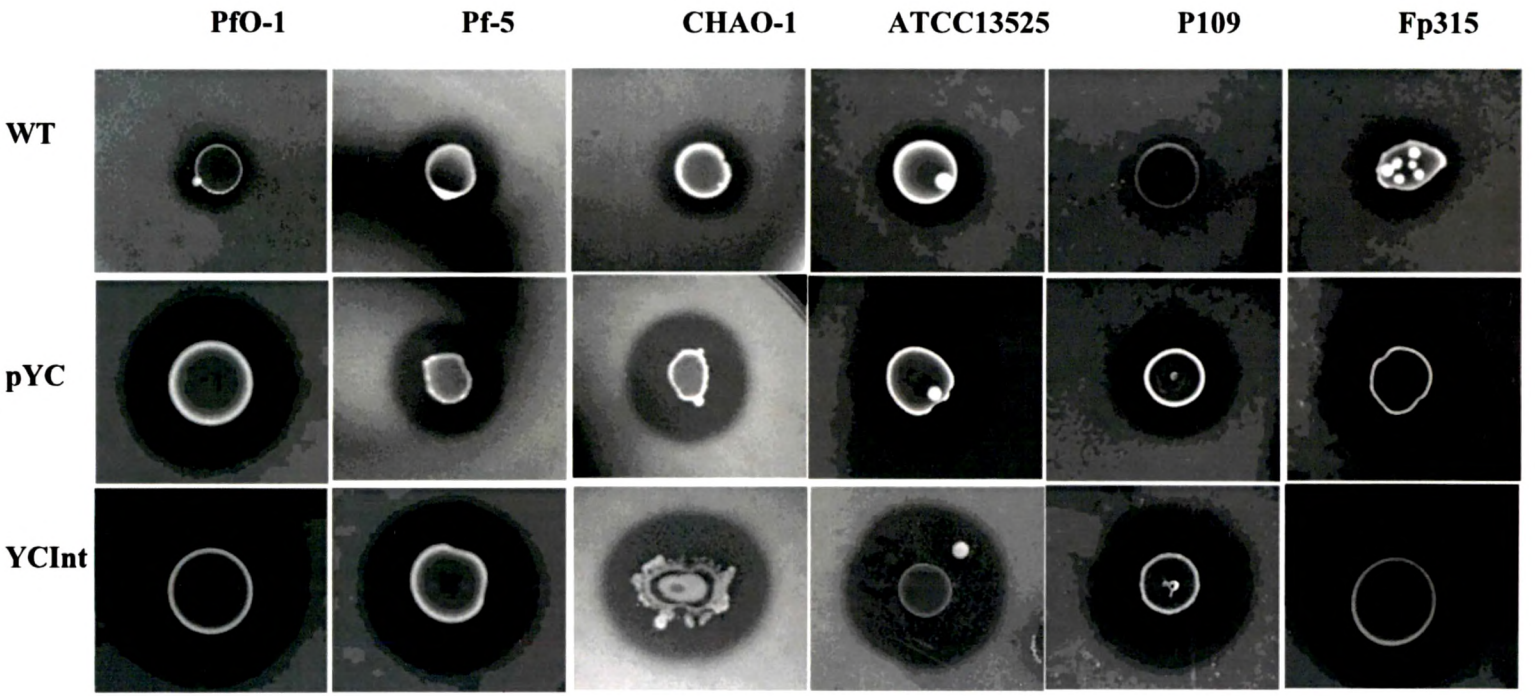
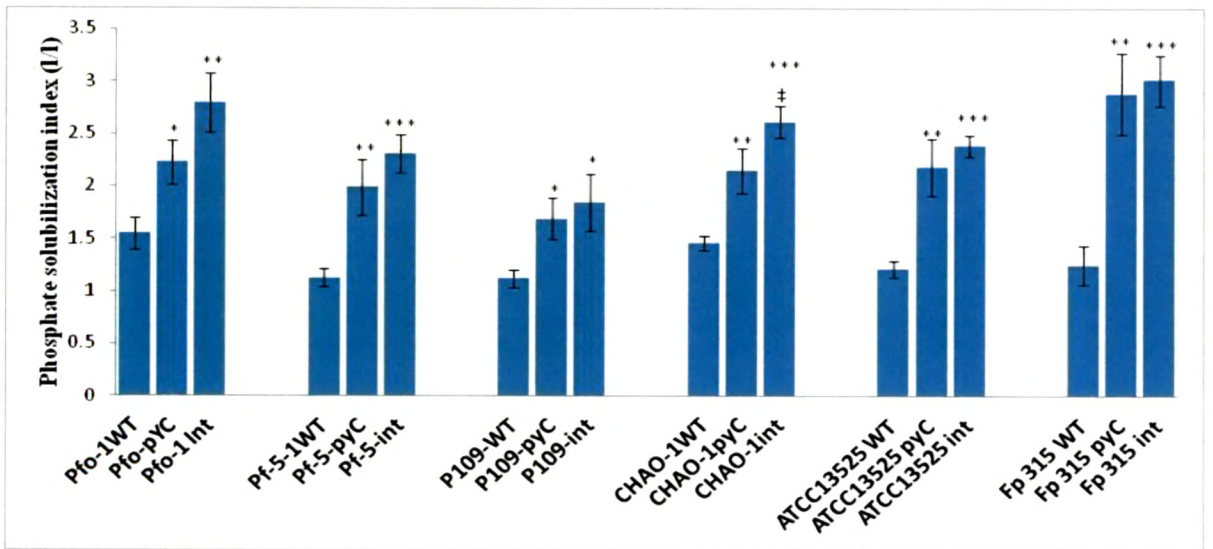
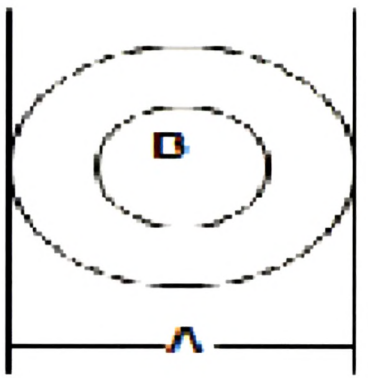


Figure 6.2: Zone of clearance of transgenic *P. fluorescens* strains in Pikovskyas agar medium during 96-120 h of growth. WT: wild type strain; Gm: *P. fluorescens with* pUCPM18Gm vector; YC: *P. fluorescens* with pYC plasmid; YCInt: *P. fluorescens* yc operon genomic integrant.





PSI= A/B

Srains	PfO-1	Pf-5	CHAO-1	ATCC13525	P109	Fp315
WT	1.55±0.15	1.13±0.09	1.46±0.07	1.22±0.08	1.12±.08	1.25±0.18
pYC	2.22±0.21	1.99±0.27	2.15±0.22	2.18±0.27	1.69±0.19	2.89±0.39
INT	2.79±0.28	2.31±0.18	2.62±0.16	2.39±0.1	1.85±0.27	3.02±0.24

Figure 6.3: P solubilization index on Pikovskyas agar of *P. fluorescens yc* operon genomic integrants during 96-120 h of growth. WT: wild type strain; YC: *P. fluorescens* with pYC plasmid; YCInt: *P. fluorescens* yc operon genomic integrant.

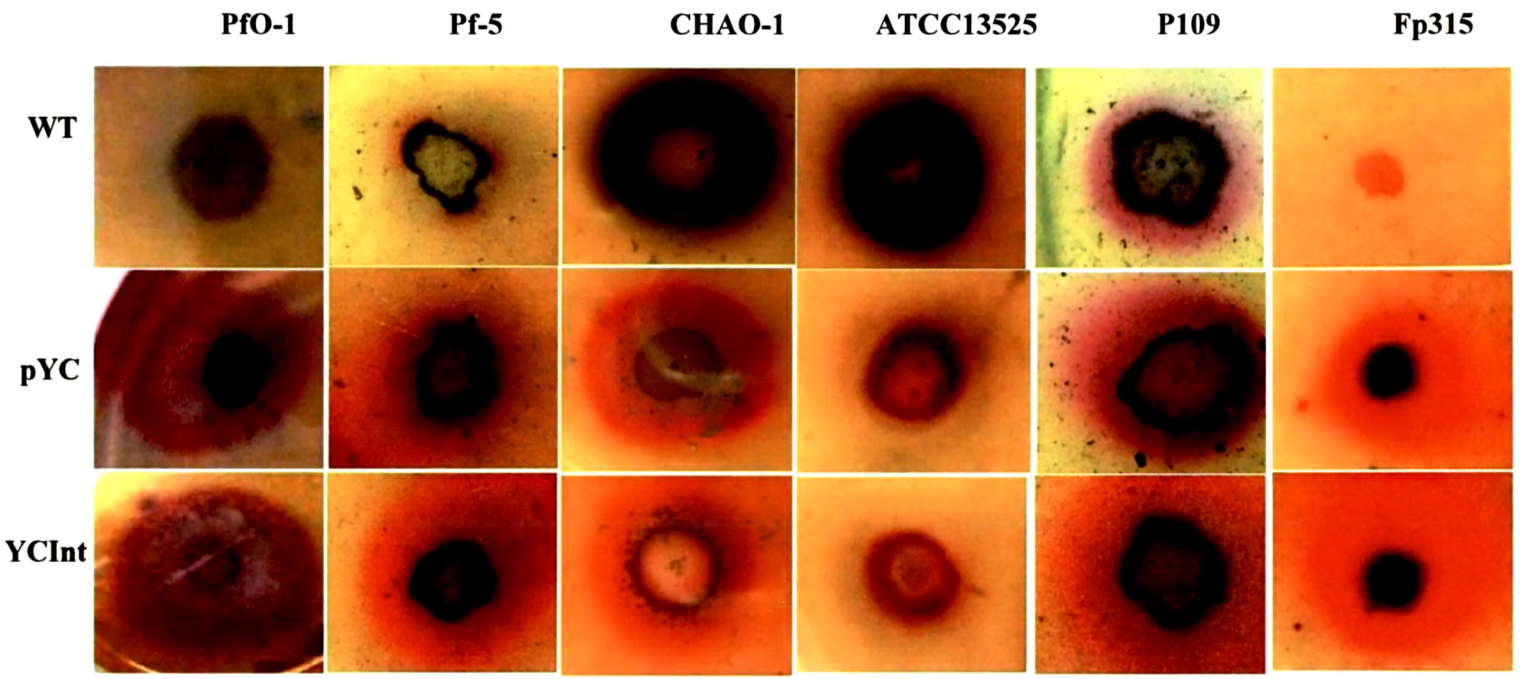


Figure 6.4: **Zone of colouration of transgenic *P. fluorescens* strains in TRP agar medium during 96-120 h of growth.** WT: wild type strain; Gm: *P. fluorescens with* pUCPM18Gm vector; pYC: *P. fluorescens* with pYC plasmid; YCInt: *P. fluorescens* yc operon genomic integrant.

6.3.1.1 P solubilization activity on buffered broth medium

All *P. fluorescens yc* operon plasmid transformants and genomic integrants showed significant enhancement of Pi release as compared to the wild type strains with an exception of P109 wherein compared to the wild type no improvement of Pi release was observed. *P. fluorescens* Fp315 genomic integrant showed a maximum Pi release of 983.4±58.78 µM which was even 1.37 fold higher as compared to plasmid transformants. In contrast, ATCC13525 genomic integrant showed a decrease in Pi level by 1.5 fold as compared to the plasmid transformants. The Pi release among the *P fluorescens* genomic integrants was in the increasing order of ATCC13525< P109 < Pf-5 < CHAO-1 < PfO-1 < Fp315 (**Fig. 6.5**).

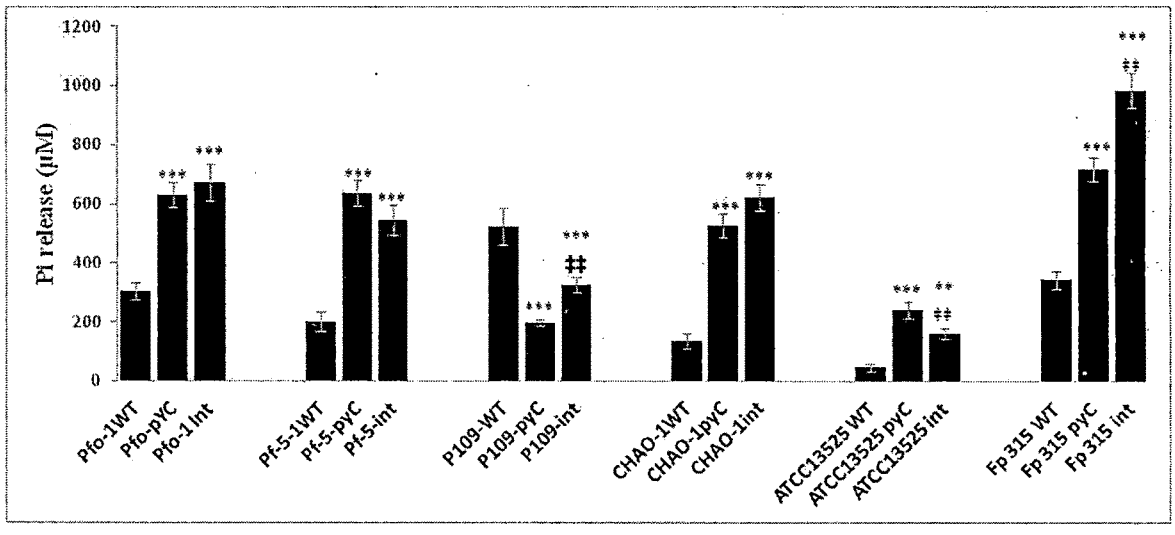


Figure 6.5: Pi release of transgenic *P. fluorescens* strains in TRP broth medium during 96-120 h of growth.

6.3.2 Greenhouse experiments

Mung bean inoculation studies in greenhouse were carried till 45 days after emergence. All growth and biochemical parameters were statistically analyzed using two factorial CRD. The results indicate that all treatments worked significantly well as compared to the UT control and many treatments were at par or significantly better as compared to the

SSP control. Analysis of variance results revealed significant influence of genomic integration on growth promotion of mung bean plants (**Appendix I**).

6.3.2.1 Growth parameters

6.3.2.1.1 Leaf number

Treatments involving inoculum of PfO-1(Int), Pf-5 (pYC), Pf-5 (Int), P109 (pYC), p109 (Int), CHAO-1 (Int), ATCC13525 (pYC), ATCC13525 (Int), Fp315 (Int) showed significant increase in leaf number and were similar to the SSP control as compared to the untreated control (**Fig. 6.6**). All treatments showed better growth as compared to the uninoculated control (**Fig. 6.7**).

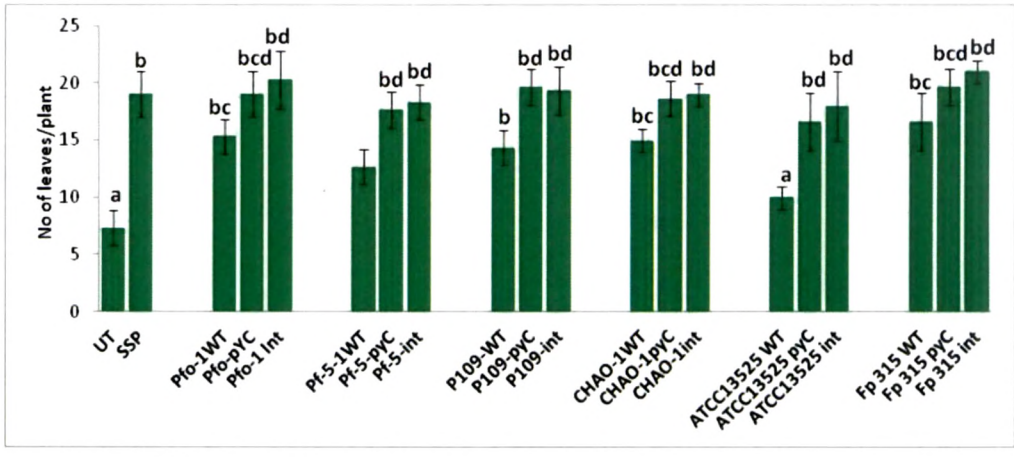


Figure 6.6: Effect of *P. fluorescens* genomic integrants on leaf number of mung bean (*Vigna radiata*) at 45 Days after sowing. Columns labeled with the same letter represents non significantly different means ($p<0.05$) after ANOVA

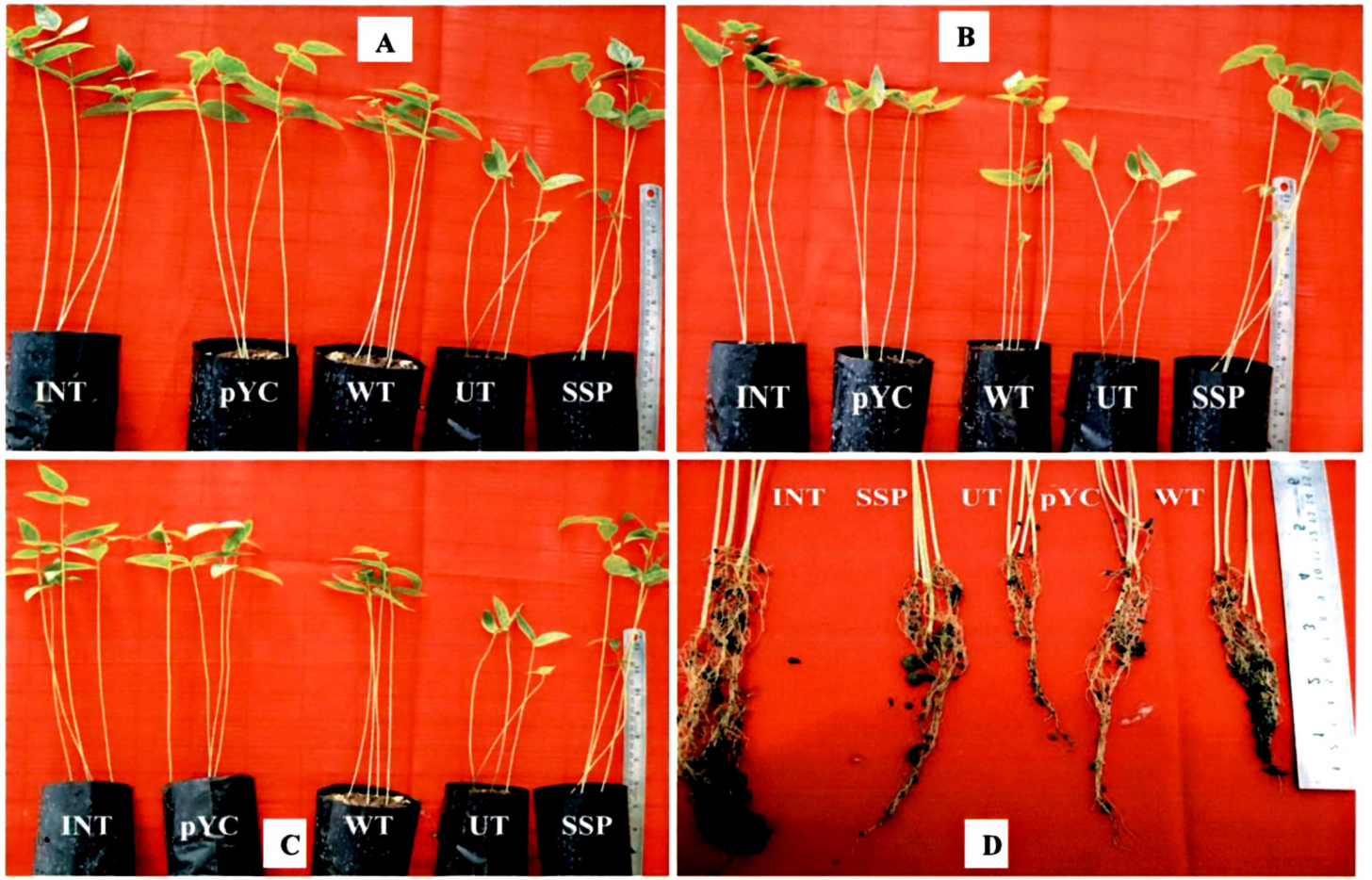


Figure 6.7: The pot results of *Vigna radiata* GM4 at 20 days after showing (DAS). The treatments are Single super phosphate (SSP) and untreated (UT) as control, Wild type (WT), yc operon plasmid transformants (pYC) and yc operon genomic integrant (INT). A: *P. fluorescens* PfO-1; B-D: *P. fluorescens* Fp315

6.3.2.1.2 Shoot and root length and dry matter accumulation

The mung bean plant inoculated with plasmid transformants and genomic integrants of all the *P. fluorescens* strains showed similar shoot length, root length, shoot dry weight and root dry weight as compared to the SSP control. All treatments showed significant improvement in shoot length and root length as compared to the untreated control except P109WT and ATCC13525WT (**Fig. 6.8**).

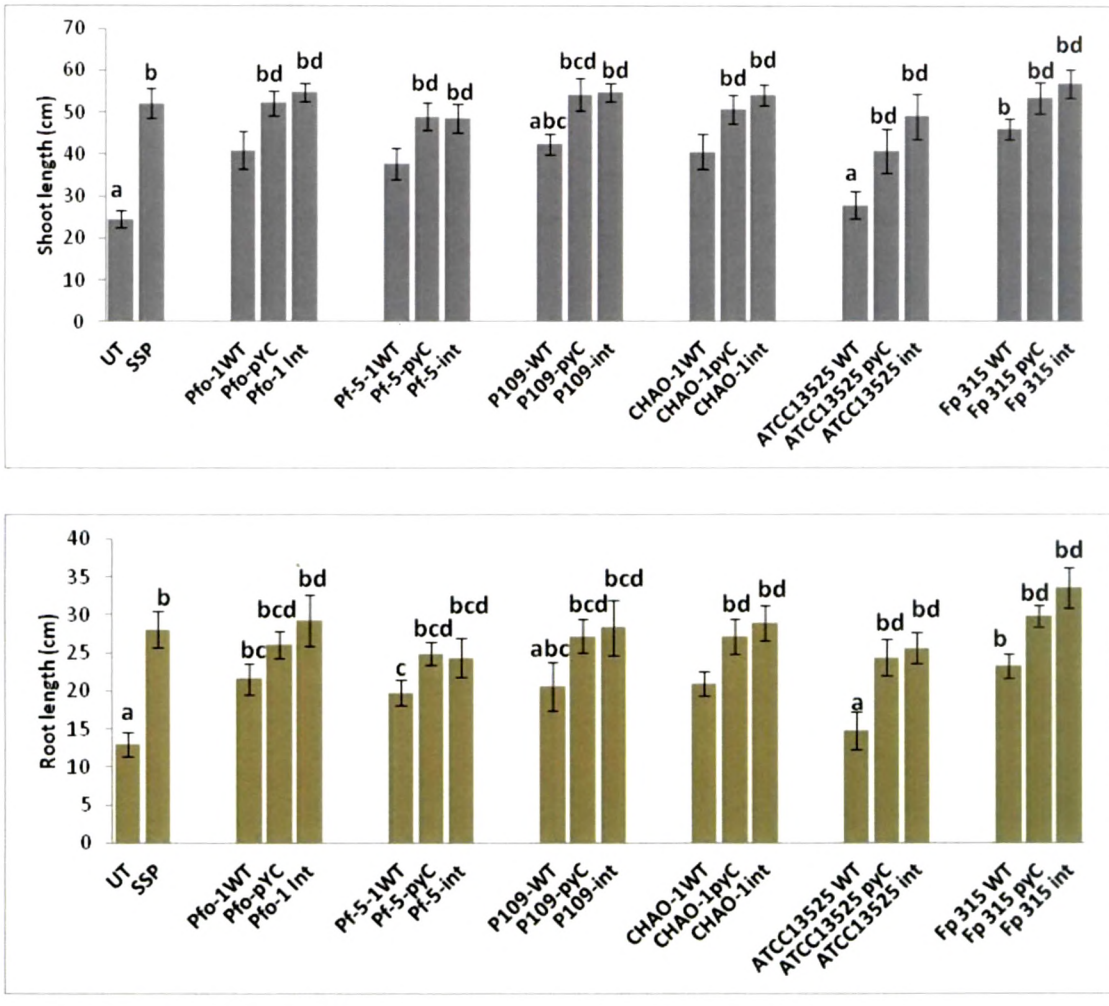


Figure 6.8: Effect of *P. fluorescens* genomic integrants on shoot length and root length of mung bean (*Vigna radiata* GM4) at 45 Days after sowing. Columns labeled with the same letter represents non significantly different means ($p<0.05$) after ANOVA

6.3.2.1.3 Dry matter accumulation

Similarly, PfO-1 WT showed no significant improvement in shoot dry weight and ATCC13525WT in both shoot and root dry weight as compared to the untreated plant (**Fig. 6.9**).

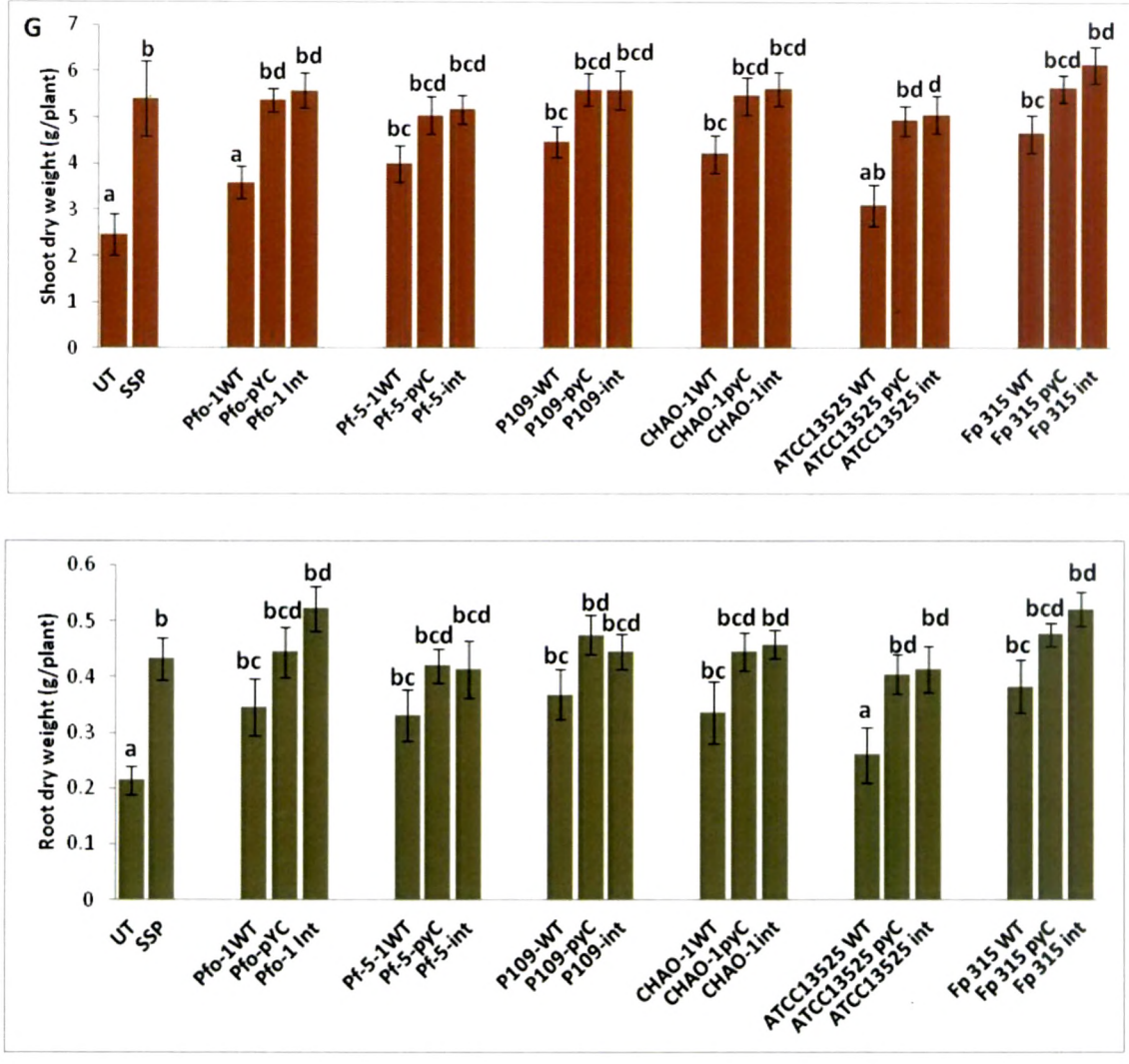


Figure 6.9: Effect of *P. fluorescens* genomic integrants on Shoot and root dry weight of mung bean (*Vigna radiata*) at 45 Days after sowing. Columns labeled with the same letter represents non significantly different means ($p<0.05$) after ANOVA

6.3.2.2 Nodulation

All the *P. fluorescens* strain *yc* operon plasmid transformants and genomic integrants showed significant increase in nodule number as compared to the untreated control and similar nodule number as compared to the SSP control. The best nodulation was observed in PfO-1(Int), Fp315 (pYC) and Fp315 (Int), nodule number of which were significantly superior to the SSP control. Similarly, nodule dry weight recorded from treatment of PfO-1 (pYC), PfO-1 (Int), P109 (pYC), P109 (Int), CHAO-1(pYC), ATCC13525 (Int), Fp315 (pYC) and Fp315 (Int) were significantly better than the SSP control (**Fig. 6.10**).

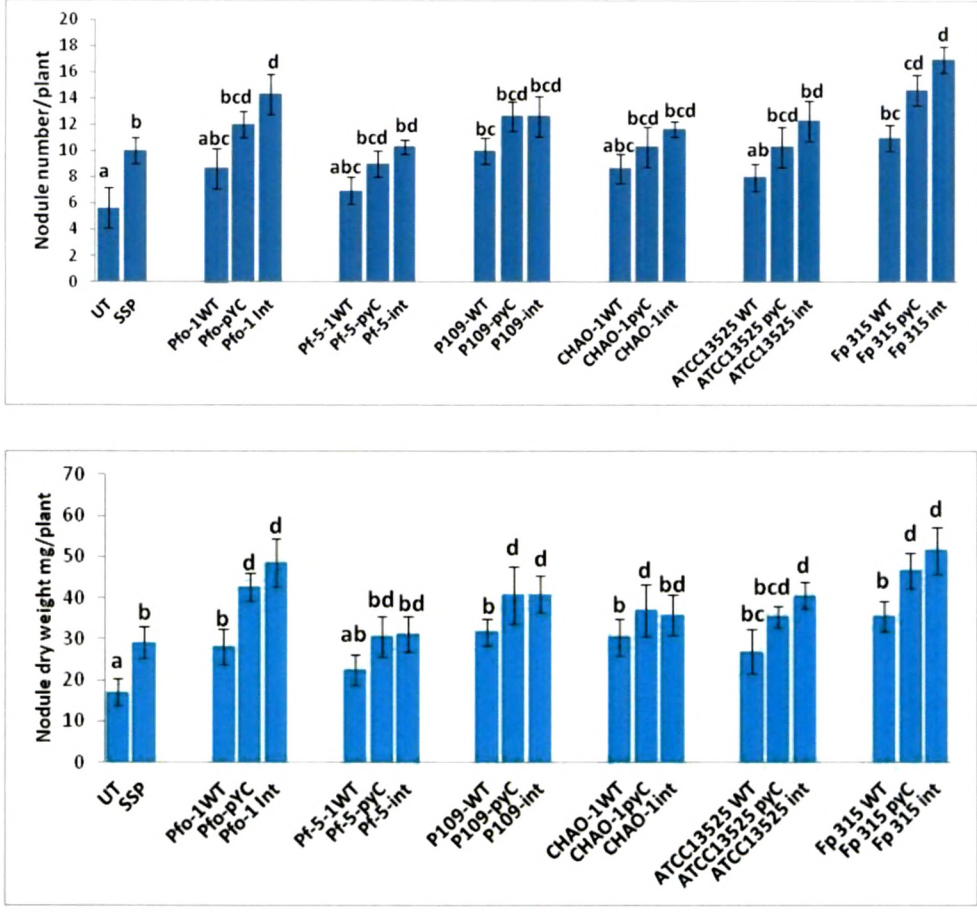


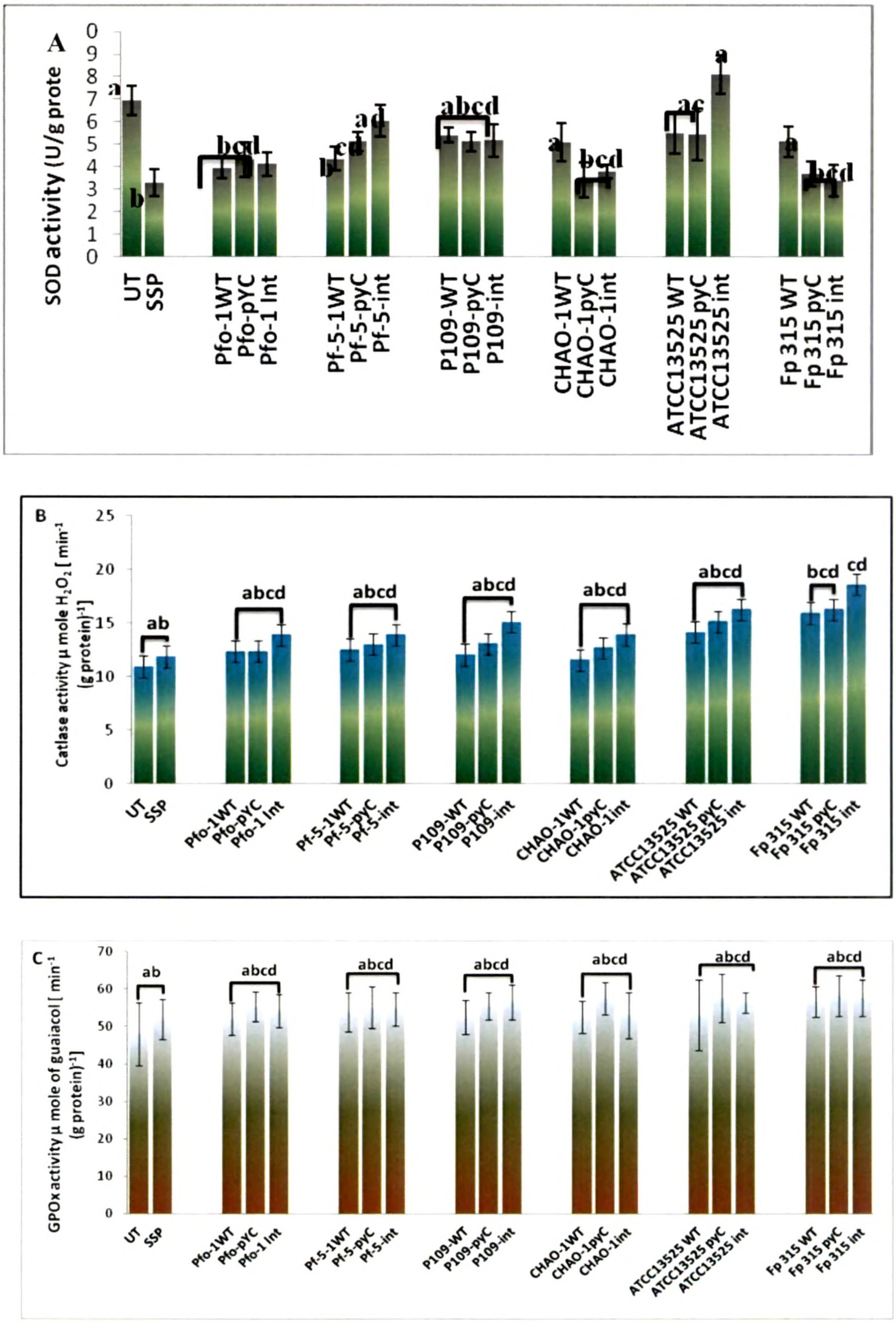
Figure 6.10: Effect of *P. fluorescens* genomic integrants on nodule number and dry weight of mung bean (*Vigna radiata*) at 45 Days after sowing. Columns labeled with the same letter represents non significantly different means ($p<0.05$) after ANOVA

6.3.2.3 Enzyme activities

To determine the effect of *P. fluorescens* genomic integrant treatment on the antioxidant status of mung bean plant, the specific activities of antioxidant enzyme **Superoxide Dismutase** (SOD), **Catalase**, **Guaiacol Peroxidase** (POX) and **Ascorbate peroxidase** (APX) were monitored at 45 days after emergence under P limiting condition. In addition, **Acid Phosphatase** (APase) and **Nitrate Reductase** (NR) activities were also determined to monitor the biochemical basis of P and N status. Significant changes in NR, APase and SOD activities were observed among all the treatments with no alterations in catalase, APX and POX activities (**Fig.6.11B-D**).

Significant reduction in SOD activities were observed in SSP control (2.1 fold), PfO-1 wild type and transgenics (~ 1.7 fold), Fp315(pYC) (1.88 fold) and Fp315(Int) (2.06 fold), CHAO-1 (pYC) (1.77) fold, CHAO-1 (Int) (2.04 fold). No alteration in SOD activities observed in p109 strains and CHAO-1 WT and Fp315 WT strains as compared to the uninoculated conrol (**Fig.6.11A**). Nitrate reductase activity was found to be repressed in uninoculated control which is 3.45 fold less as compared to the SSP control. Mung bean inoculated with all the *P. fluorescens* PfO-1 (int), Pf-5 (Int) and Fp315 (Int) showed highly significant increase in NR activity as compared to the uninoculated control. Except ATCC13525 WT and Fp315 WT all other wild type strains did not show significant improvement of NR activity (**Fig.6.11E**).

The APase activity of all the *yc* plasmid transformants and genomic integrant strains inoculated seedlings was significantly higher than uninoculated control. The wild type culture of CHAO-1 and Fp315 showed an increase by 2.17 fold and 2.2 fold, respectively, in APase activity as compared to the uninoculated control. The highest APase activity was found in Fp315 (Int) which is 3.6 fold and 1.46 fold higher as compared to the uninoculated and SSP control, respectively. PfO-1(Int) also showed a higher (1.4 fold).APase activity compared to the SSP control.


A
SOD activity (U/g prote
UT
SSP
Pfo-1WT
Pfo-pYC
Pfo-1 Int
Pf-5-1WT
Pf-5-pyC
Pf-5-int
P109-WT
P109-pyC
P109-int
CHAO-1WT
CHAO-1pyC
CHAO-1int
ATCC13525 WT
ATCC13525 pyC
ATCC13525 int
Fp 315 WT
Fp 315 pyC
Fp 315 int
B
Catlase activity µ mole H2O2 [min-1 (g protein)-1]
C
GPOx activity µ mole of guaiacol [min-1 (g protein)-1]
ab
abcd
bcd
cd

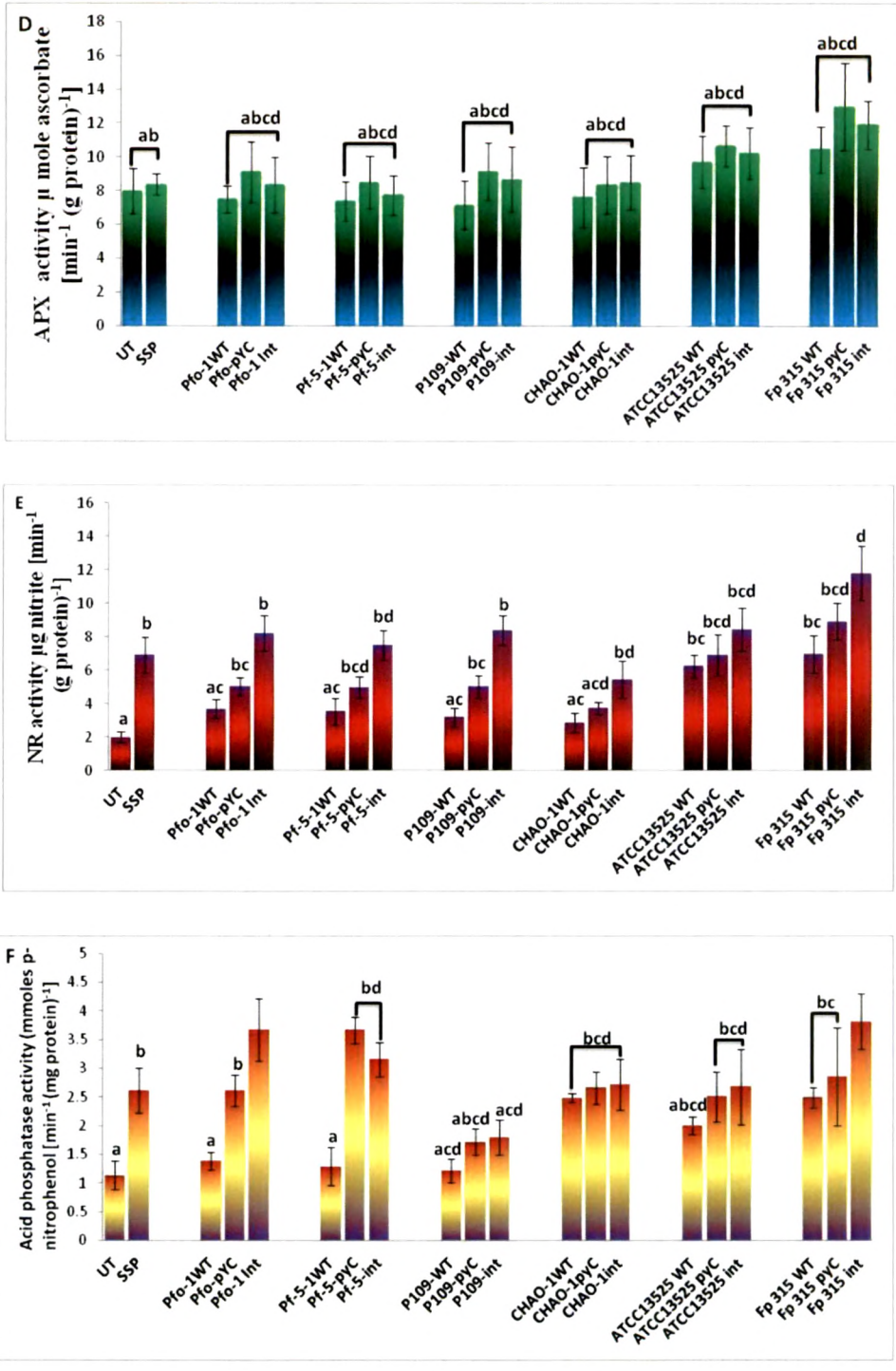


Figure 6.11: Effect of *P. fluorescens* genomic integrants on enzyme activities of mung bean (*Vigna radiata*) at 45 Days after sowing. Columns labeled with the same letter represents non significantly different means ($p<0.05$) after ANOVA

6.3.2.4 P content in shoot and root

Significant increase in root P content was observed in both plasmid transformants and genomic integrant of ATCC13525 and CHAO-1 and genomic integrants of PfO-1 and Fp315. Highest root P content was observed in Fp315(Int) which is even more than SSP control (**Fig.6.12**).

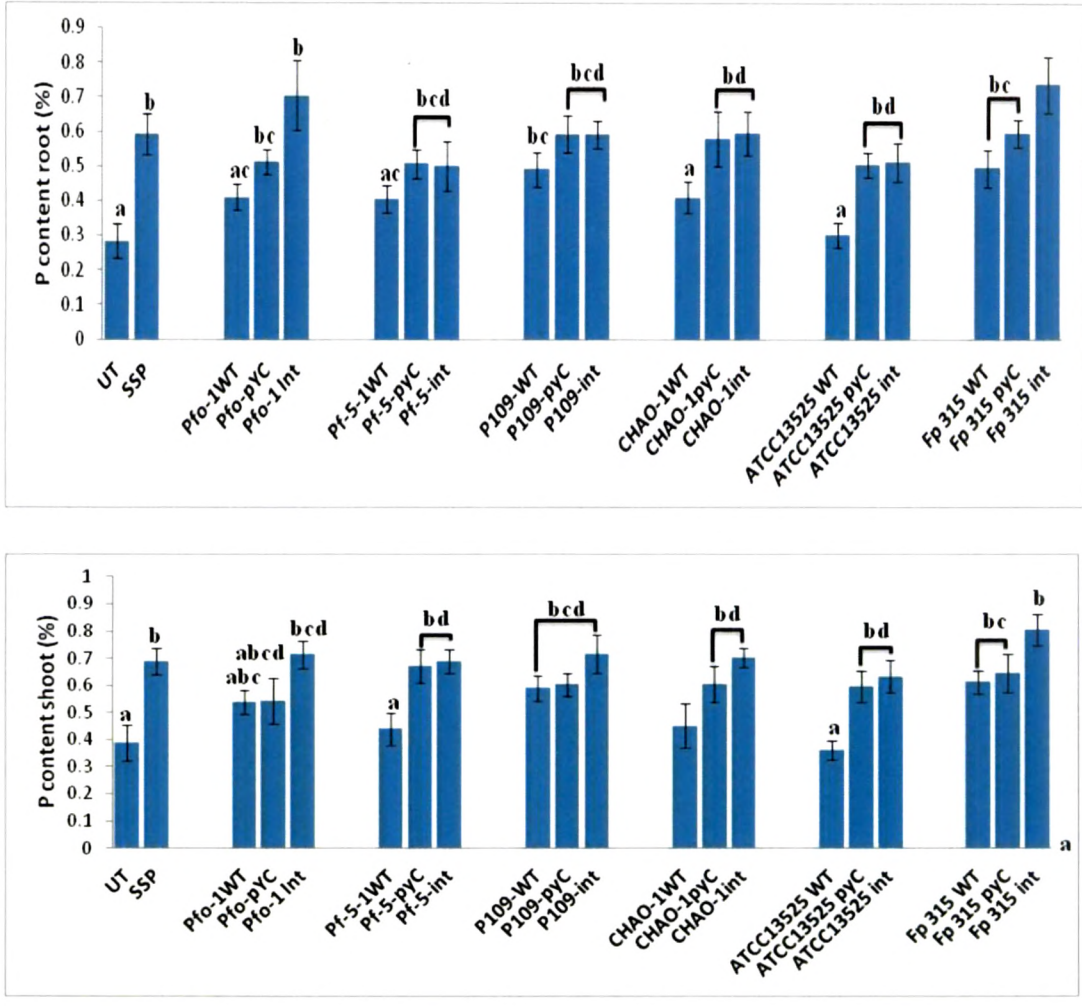


Figure 6.12: Effect of *P. fluorescens* genomic integrants on P content of root and shoot of mung bean (*Vigna radiata*) at 45 Days after sowing. Columns labeled with the same letter represents non significantly different means ($p<0.05$) after ANOVA

6.3.2.5 Available Soil P

Inoculation with PfO-1, Pf-5 and Fp315 increased soluble P as compared to the uninoculated control. The wild type strains of ACCC13525, CHAO-1 and P109 did not result in increase soil Pi.

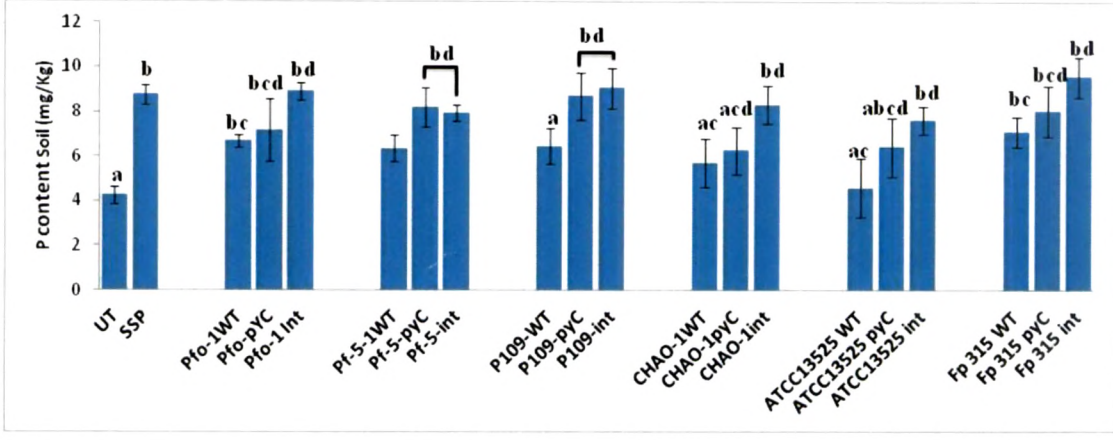


Figure 6.13:. Effect of *P. fluorescens* genomic integrants on available soil phosphorous of mung bean (*Vigna radiata*) at 45 Days after sowing. Columns labeled with the same letter represents non significantly different means ($p<0.05$) after ANOVA

6.4 Discussion

The present work describes the effect of *yc* genomic integration on MPS ability of fluorescent pseudomonads including natural isolates on plan growth promotion of mung bean (*Vigna radiata* GM4) in phosphate deficient soil under controlled environment of green house. P solubilization index of transgenic *P. fluorescens* PfO-1 on PVK agar medium was in the order of WT≤ Gm< YF<YC≤ YCInt and gave phenotype in TRP medium containing 75mM tris HCl and 75 mM glucose, demonstrating the improved functionality of the *yc* operon genomic integration. Among PSMs, only few *Bacillus* strains solubilize mineral phosphates via citric acid secretion. But higher citric acid secretion has been demonstrated in MPS fungi belonging to *Aspergillus* and *Penicillium* spp. (Khan et al., 2006). Genetic modification strategy was investigated in diverse fluorescent pseudomonads

including *P. fluorescens* PfO-1, Pf-5, CHAO-1, ATCC13525, P109, and Fp315. All these pseudomonads are noted for their metabolic diversity and are being investigated extensively for use in applications such as bioremediation of various organic compounds viz., styrene, TNT and, polycyclic aromatic hydrocarbons, and biocontrol of pathogens in agriculture (CHAO-1, Pf-5, PfO-1). These *P. fluorescens* strains, protect plant roots against soil born pathogens such as *Fusarium* or *Pythium*, as well as phytophagous nematodes through induce systemic resistance in the host plant, outcompete pathogenic soil microbes by siderophores giving a competitive advantage at scavenging for iron, produce compounds antagonistic to other soil microbes, such as phenazine-type antibiotics, secondary metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) and/or hydrogen cyanide. All strains are also known for plant growth promotion to varying extent in different host plants (Ref). *P. fluorescens* P109 and Fp315 are wheat rhizospheric isolates with poor P solubilizers. Such bacteria would be really advantageous if possess efficient MPS ability along with potent biocontrol abilities, thereby serve the current demand for multipurpose biofertilizers (Vessey et al., 2003; Vassilev et al., 2006). Our laboratory results showed improvement of MPS ability in all the strains genomic integrant as compared to wild type strain The MPS phenotype in both the unbuffered and Tris HCl buffered media can be correlated with increase in gluconic and citric acid and the amount of Pi release except P109 strain. The wild type strain of P109 showed higher amount of Pi release as compared to the transgenic strain.

Genetically modified fluorescent pseudomonad strains were tested for growth response of mung bean in phosphate deficient soil condition in controlled environment of green house. All the transgenic strains showed enhancement of all plant growth parameters (leaf number, plant height and dry weight, nodule number, and dry weight) and remarkably at par with the SSP control. *P. fluorescens* ATCC13525 wild type strain showed no significant enhancement in growth promotion compared to the untreated control and growth promotion of other wild type strain was at par or less than the SSP control. The effect of genomic integration of *yc* operon in the bacteria clearly increased the nodule number and dry weight more than native strains and in case of PfO-1 and Fp315, increase was even better than SSP control. Improvement in nodulaion due to inoculation of P solubilizers was

also reported in chick pea and other leguminous plant (Tang et al., 2001). Enhanced nodulation after inoculation of the strain suggested an increase in available P for the plant as leguminous plant require high amount of P for nodule formation and mainainance of high rate of bacterial activity inside the nodule (Leidi et al., 2000; Zaman et al., 2007). The increase dry matter accumulation following inoculation of PSM correlated to increased nodulation, increased shoot and root length and increased number of leaves per plant. The P content of shoot was increased after inoculation with *P fluorescens* strains. Similar results obtained in barley and chick pea when grown in soil treated with insoluble phosphate and PSM *Mesorrhizobium mediterraneum* PECA21 where the P content was significantly increased by 100 and 125%, respectively as compared to control (Peix et al., 2001).

Present study showed the decrease in SOD activity but not in catalase and total peroxidase activities after inoculation with *P. fluorescens* PfO-1 WT and transgenic strain of Fp315, CHAO-1 and Pf-5. Inoculation of PGPR reported to reduce oxidative stress in plants. Abiotic stress conditions cause an increase in ROS formation such as superoxide radical (O^{2-}), hydrogen peroxide, and hydroxyl radicals (OH) at the cellular level (Sgherri and others 2000; Hemavathi and others 2010). Al toxicity and P deficiency both increased SOD and POD activities in maize and rice plants (Tewari et al, 2004; Sharma and Dubey, 2007). Bacterial inoculation benefits plant physiology and growth under abiotic stress conditions including nutrient deficiency (Carmen et al., 2011). Induction of antioxidant enzymes (catalase. SOD, APX, GR and POX) is involved in the alleviation of salinity stress in lettuce plants inoculated with PGPR strains (Bianco & Defez, 2009; Kohler et al., 2010). In contrast, PGPR inoculated plants showed significantly lower activity of antioxidant enzymes as compared to uninoculated plants (Omar et al.,2009; Sandhya et al. 2010). Significant increase of catalase and peroxidase activities is found in salt-stressed leaves of two barley cultivars differing in salinity tolerance after inoculation with *Azospirillum brasilense* (Omar et al., 2009). In contrast, the mRNA expression of SOD CAT, DHAR, GR and APX in bacteria-inoculated considerably increased in plants grown under stress conditions when compared with that of noninoculated stressed plants (Gururani et al 2012).

Plant growth promotion has been associated with the solubilization and increased uptake of phosphate. In the present study, phosphorus use efficiency of mung bean and soil quality were improved after PGPR application as indicated by increased APase activities and soil Pi content. APase induction and nitrate reductase repression is a significant adaptive response under Pi limited condition. Simultaneous exudation of organic acids and APase could increase both P solubility, by releasing bound organic phosphates and its mineralization by increasing the rate of hydrolytic cleavage (George et al., 2002). In addition, nitrate reductase is found to be repressed by Pi starvation, indicating that nitrogen assimilation is repressed under Pi depletion (Wu et al., 2003; Gniazdowska and Rychter, 2000). In the present study, significant enhancement of nitrate reductase activity was observed after inoculation with *P. fluorescens* genomic integrants as compared to the uninoculated plants grown under Pi limited condition. In bean (*Phaseolus vulgaris*), Pi deprivation leads to increased root nitrate accumulation and suppresses shoot nitrate accumulation, possibly by repressing nitrate reductase activity and nitrate transport from roots to shoots. A similar alteration in nitrate contents was observed in wild-type rice plants under Pi starvation conditions, indicating that rice also represses nitrate assimilation during Pi starvation. (Gniazdowska and Rychter, 2000; Hu et al., 2011).

Summarizing, present study demonstrates significant improvement in mung bean growth by transgenic *P. fluorescens.* Another important feature of the present study, the plant inoculation experiments were carried with the genomic integrants in soils without supplementation of rock phosphate. Thus, these bacteria appear to provided mung bean P derived from soil (alkaline vertisol) phosphate. All treatments, showed a consistent increase in growth response to the same level as the SSP control or in some cases even better. Further, improvement in some growth parameters was better in genomic integrants than plasmid transformants. In conclusion, the present study demonstrates that the genetic modifications of diverse fluorescent pseudomonads have similar effects in the central metabolism leading to secretion of citric acid to similar level.