Chapter 8

Effect of *Sinorhizobium fredii* NGR 234 genomic integrant containing *E. coli* NADH insensitive *cs* and *S. typhimurium citC, vgb* and *egfp* gene cluster on growth promotion of Mung bean (*Vigna radiata*) plants

8.1 Introduction

Legumes are second only to cereals as a source of human food and animal feed. Their importance as food lies primarily in their high protein content. Legumes' grain protein is the natural supplement to cereal grain protein. They also provide fat and carbohydrates. Moreover, legumes are high in bone building minerals and vitamins essential for good health (Porres *et al.*, 2003). Many efforts were directed to improve yield and protein content of legumes through breeding, fertilization and genetic engineering. Biofertilizers are inputs containing microorganisms which are capable of mobilizing nutritive elements from non-usable form to usable form through biological processes; they include mainly the nitrogen fixing, phosphate solubilizing and plant growth–promoting microorganisms (Goel *et al.*, 1999). 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).

The inoculation of seeds with *Rhizobium* increased nodulation, nitrogen uptake, seed protein (Solaiman, 1999; Rudresh, 2005). *Rhizobium* inoculant significantly increased number of pods, nodule dry weight compared to uninoculated control in chickpea (Solaiman ,1999; Daza et al., 2003; Rabbani et al., 2005; Togay et al., 2008). The combined inoculation of *Rhizobium* and phosphate solubilizing bacteria has increased nodulation, plant height, seed protein and yield parameters in chickpea [Khurana and Sharma, 2000; Namvar et al., 2011). Co-inoculation with nodule-forming and rhizospheric soil bacteria improved growth and nodulation through multiple mechanisms (Contesto et al. 2008; Zadeh et al. 2008). Several PGPR produced ACC-deaminase (e.g. Dimkpa et al. 2009; Shaharoona et al. 2012) and thus modulated C_2H_4 levels in plant through the cleavage of ACC (Glick et al. 1997).

The conducive effect of dual inoculation of seeds with *Rhizobium* and microelements application on nodulation and N_2 fixation in legumes has been established (Zehirov and Georgiev, 2001). Inoculation of seed with *Rhizobium* in combination with microelement fertilizer significantly affected the yield and nodules formation of chickpea (Bejandi et al., 2012). Concentration of chlorophyll dyes is a reliable index of physiological plant condition (Swedrzynska and Sawicka, 2000). However, little work has been done on the combined effects of *Rhizobium* inoculation, microelements application and plant density on nodulation, seedling emergence, chlorophyll content, seed protein and grain yield.

After nitrogen, phosphorus is generally the most limiting nutrient affecting plant growth and productivity. In legumes, phosphorus availability stimulates the nodulation and N_2 fixation since nodule initiation and nodule growth require P (Gentili and Huss-Danell 2003). Phosphorus deficiency generally results in impaired symbiotic N fixation by limiting plant growth, survival of rhizobia, nodule formation, and nodule functioning (Tang et al. 2001; Bargaz et al. 2011). Low organic matter content coupled with native low soil P pool is a major factor limiting agricultural productivity in the Indian subcontinent (Manna et al. 2001). Soil organic amendments increased soil biological activity and improved physical and chemical properties (Gaind et al. 2006). Addition of organic amendment to soil stimulated microbial activity that in turn increased organic matter decomposition rate and nutrient dynamics (Abbott and Murphy 2007; Chakraborty et al. 2011). Microbial biomass has vital role in regulating nutrient source sink. Soil enzymatic activities are key determinants of soil available nutrient pools including N, P, and K (Sinsabaugh et al. 2009; Nannipieri et al. 2012).

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 (Ahmad and Khan, 2011). 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). 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; Rodríguez and Fraga, 2006; Khan et al., 2007; Harvey et al., 2009; Khan et al., 2010). But, the effectiveness of PSMs in the laboratory or controlled conditions is not 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 is 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,b; 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). Inoculation of *Sinorhizobium cicero* and *Pseudomonas sp.* with 18/20 kg NP ha^{-1} as urea and di-ammonium phosphate increased nodule number, nodule dry weight, nodule volume and dry matter compared to uninoculated control at mid flowering stage in chickpea plant (Messele and Pant, 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 are 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 (Bianco and Defez, 2009; 2010a,b; Imperlini et al., 2009). Hence, genetic modification of native strains may help to survive, adapt and function better in the rhizosphere and improve plant nutrition.

Plant growth promoting rhizobacteria (PGPR) stimulated or inhibited nodule formation in a given symbiotic association, depending upon the type, nature, and concentration of secondary metabolites produced by the rhizobacteria. PGPR carrying ACC-deaminase enzyme intrinsically improved symbiotic efficiency of legumes by lowering the plant ethylene level that inhibited nodulation process (Nascimento et al. 2012).

Over-expression of genes involved in soil inorganic phosphate solubilization in natural PGPR improved 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.

MPS ability of the bacterial transformants in field conditions could be affected by various parameters. In addition to the nutrient availability and soil properties, oxygen limitation could be a significant factor. Oxygen is present in limited amounts in the rhizosphere which limits the colonization and survival of rhizobacteria (Ramírez et al.,

1999). The obligate aerobic bacterium, *Vitreoscilla*, synthesizes elevated quantities of homodimeric hemoglobin (VHb) under hypoxic growth conditions which improved growth under microaerobic conditions when dissolved oxygen is less than 2% of air saturation (Khosla and Bailey, 1988a; b). Expression of *vgb* gene encoding VHb protein in heterologous hosts often enhanced growth and metabolism by facilitating oxygen transfer to the respiratory membranes (Stark et al., 2011). Genetically modified *Rhizobium* with *vgb* overexpression has enhanced the ATP, ADP, NADH and NADPH generation, plasmid bearing *vgb* gene generated 0.624 nmoles of ATP, which significantly enhanced the nitrogenase activity up to ~170 μmol at low concentration of oxygen, while nitrogen content was increased up to ~56 mg/plant in bean plants (Ramirez et al., 1999).

In addition to the metabolic alterations in the central carbon pathway, overexpression of *vgb* gene in *Rhizobium* spp. along with artificial citrate operon improved the recombinant protein production and better survival of host under microaerobic conditions (Frey and Kallio, 2003). The MPS ability was lost when grown without an air in case of *Enterobacter intermedium* which secreted 2-ketogluconic acid (Hwangbo et al., 2003). A similar loss of MPS ability under low aeration conditions was seen in case of *Citrobacter* sp. DHRSS containing citrate operon with a concomitant loss of citric and gluconic acid secretion. Presence of *vgb* gene restored the citric and gluconic acid secretion along with MPS ability under microaerobic conditions (Yadav, 2013).

Plant growth promoting rhizobacteria (PGPR) are soil bacteria that benefited plant growth by different mechanisms (Glick, 1995; Archana et al., 2012), and P-solubilization ability of the microorganisms is one of the most important traits. The effect of genomic integration of *yc* operon containing *E. coli* NADH insensitive *cs* and *S. typhimurium citC, vgb* and *egfp* gene cluster in fluorescent pseudomonad 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 **(Fig. 8.1; 8.2; 8.3)** (Adhikary et al., 2012).

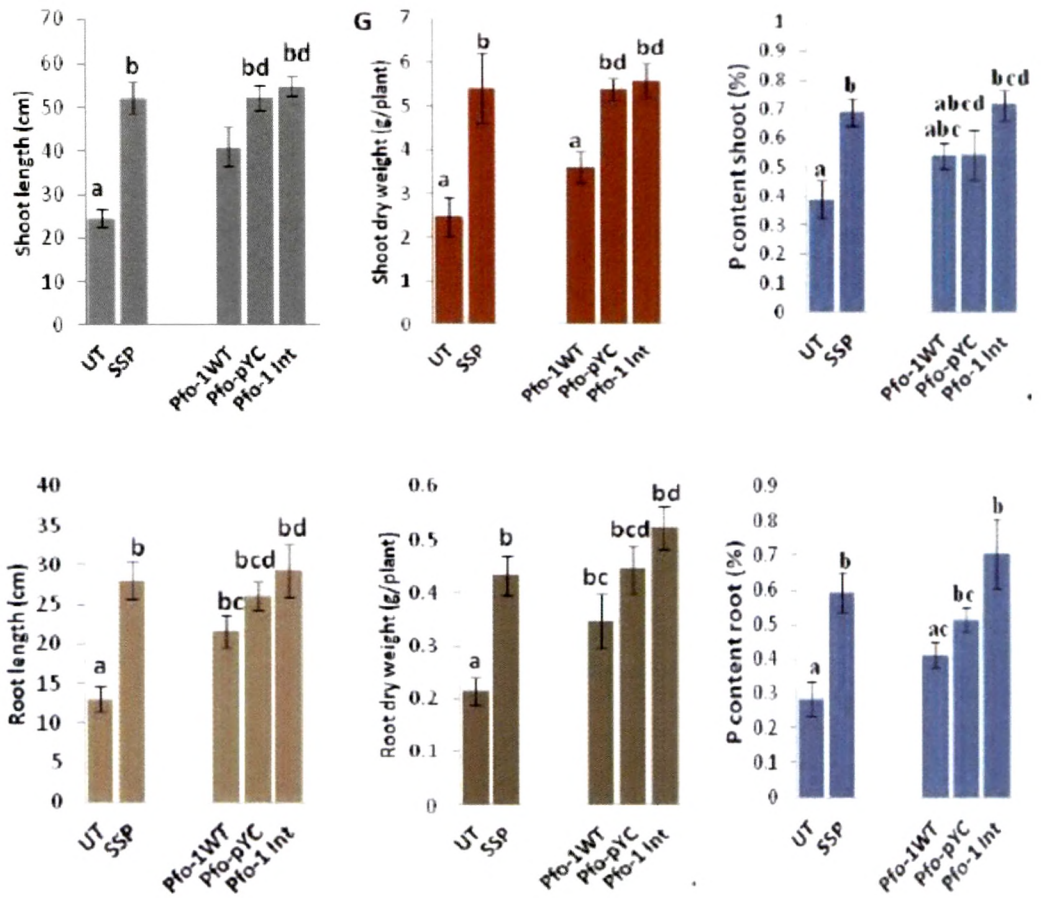


Fig. 8.1: Effect of *P. fluorescens* genomic integrants on shoot length, weight and P content and root length, weight and P content of mung bean (*Vigna radiata*) at 45 Days after sowing.

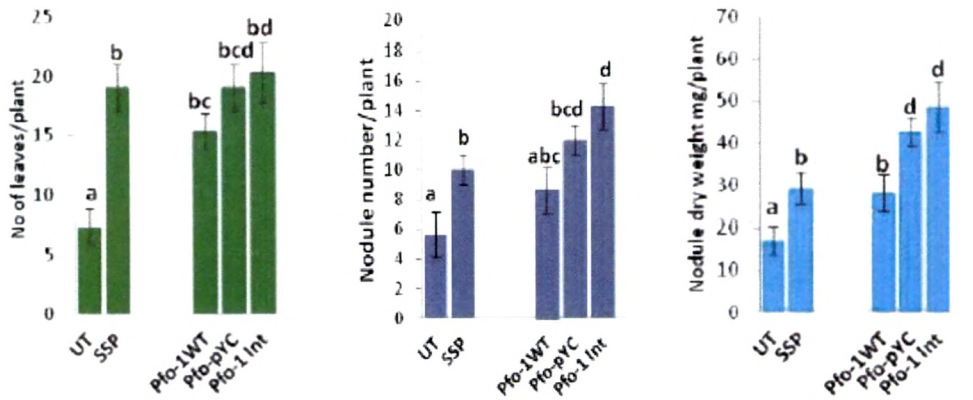


Fig. 8.2: Effect of *P. fluorescens* genomic integrants on number of leaves, nodule number and weight of mung bean (*Vigna radiata*) at 45 Days after sowing.

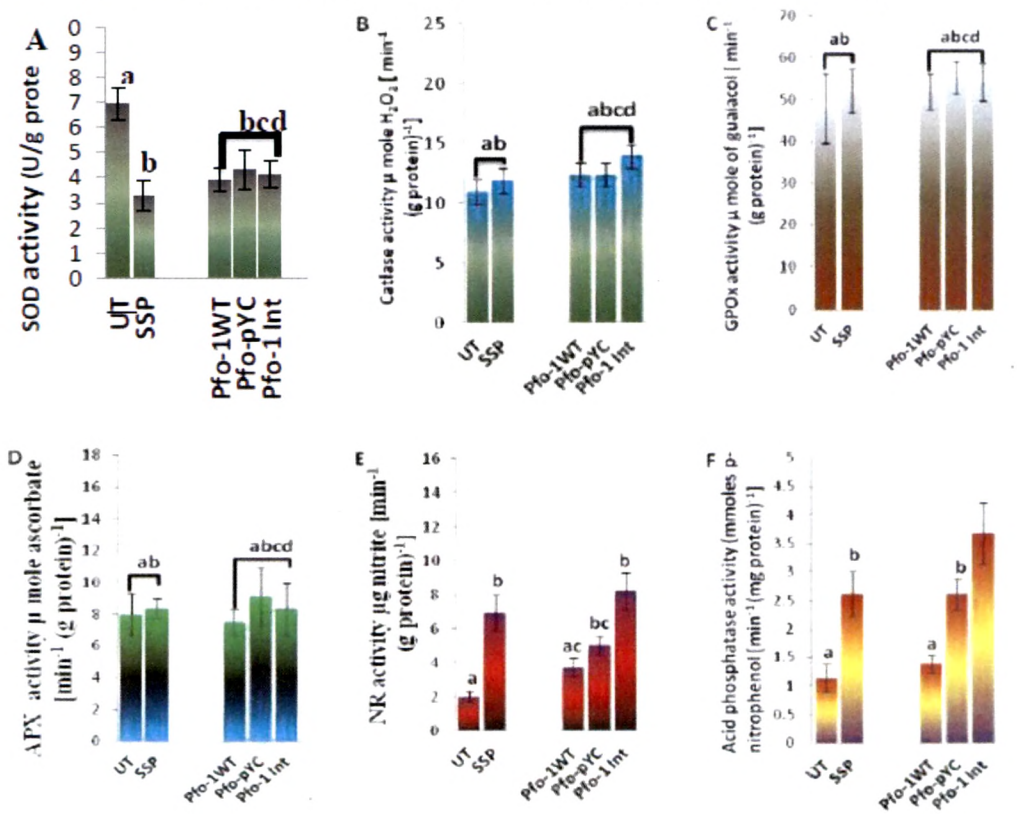


Fig. 8.3: Effect of *P. fluorescens* genomic integrants on enzyme activities of mung bean (*Vigna radiata*) at 45 Days after sowing.

8.1.2: Rationale of the Study

Metabolic 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, *Sinorhizobium fredii* NGR 234 was subjected to similar genetic modification for citric acid secretion leading to P solubilization and plant growth promotion. *S. fredii* NGR234 having wide host range for nodulation and its integrant gave better results compared to the integrants of *Bradyrhizobium japonicum* USDA110 and *Mesorhizobium loti* MAFF030669. Thus, *S. fredii* NGR234 has been selected to monitor its efficacy in providing P and promoting the growth of mung bean (*Vigna radiata*) plants. The objectives of this study were to determinate the effects of *S. fredii* integrant on rhizospheric soil, plant height and weight, leaf number and area, chlorophyll content, number and size of nodules, number and size of pods and grain yield, and determine the nitrogenase and antioxidant enzyme activities of mung bean.

8.2 Experimental design

8.2.1: Bacterial strains used in this study

Table 8.1: Bacterial strains used in this study.

Plasmid/Strains	Characteristics	Source or Reference
S. fredii NGR 234	NC_012587.1	
Sf intYc	Genomic integrant of *S. fredii NGR 234* containing *lac-YF citC, vgb, egfp* Ap^r	This study Chapter 7

8.2.2 Plant Inoculation Experiments

Pure bacterial cultures were grown in nutrient broth at 30°C, centrifuged, and diluted to a final concentration of 10^8 CFU/ml in sterile distilled water. Seeds of mung

bean obtained from local market 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. Seeds were treated with *Rhizobium* strains each containing native strain (N), and *yc* operon genomic integrant (Int). In the experiment, one control was used, where no inoculum was added and designated as control (C).

8.2.3 Greenhouse experiment

Bacteria coated Mung bean seeds were sown in pots 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 out at 20 days and 45 days after sowing and biochemical characterization was carried out at 45 days after emergence. Each treatment had 5 replications (6 seeds per replicate).

8.2.4 Growth parameter assessment

All the plant growth parameters were estimated at 20 days and 45 days after sowing (DAS).

8.2.5 Biochemical characterization

Superoxide dismutase (SOD), Catalase (CAT), Ascorbate Peroxidase (APX), Guaicol Peroxidase (POX) and nitrogenase enzyme activities were estimated at 45 days after sowing (DAS).

8.2.6 PQQ determination

PQQ production was estimated using the method of Rajpurohit et al. (2008). Fresh leaves and nodules were crushed using liquid nitrogen. Acetonitrile 50% was added

to powder and kept for digestion at 65°C for 2 h. The mixture was centrifuged at 15,000 *g* for 10 min; the clear supernatant was collected and dried with a concentrator under a vacuum. The residue was dissolved in 50% *n*-butanol at 1 mg/ml, and PQQ was extracted at 50°C overnight. The clear supernatant was dried under a vacuum and dissolved in 100% methanol. The identity of the PQQ was ascertained by comparing with standard PQQ on spectrofluorometer. Fluorescence was monitored at *ex* 360 and *em* 480 nm.

8.2.7 Isolation of bacteria from rhizospheric soil and nodules.

Bacteria isolated from rhizospheric soil and nodules from mung bean plants of 45 days and were isolated on agar plate with appropriate dilutions. Further, total bacterial count was mentioned in CFU.

8.2.8 Statistical analysis

The experiments were carried out in a completely randomized design (CRD) for mung bean. The experimental data was analyzed statistically using Prism 3.

8.3 RESULTS

8.3.1 Effect of genomic integrant on bacteria of rhizospheric soil and bacteroids of nodules.

Fluorescent colonies were not seen in the control and native plate but were seen in experimental plate under UV light in the samples isolated from rhizospheric soil. Mucoid colonies of *Rhizobium* were seen in the control and native plates and fluorescent colonies of *S. fredii* NGR 234 with the integrant were seen in the plate containing isolate from nodules of nodules from the inoculated mung bean plant (**Fig. 8.4**). There was increase by 24 fold and 150 fold in CFU count from the rhizospheric soil and bacteroids from nodules of mung bean plant, respectively, inoculated with the integrant (**Table 8.2**).

Weight of nodules increased by 3.3 fold of the integrant treated plant as compared to the control plant.

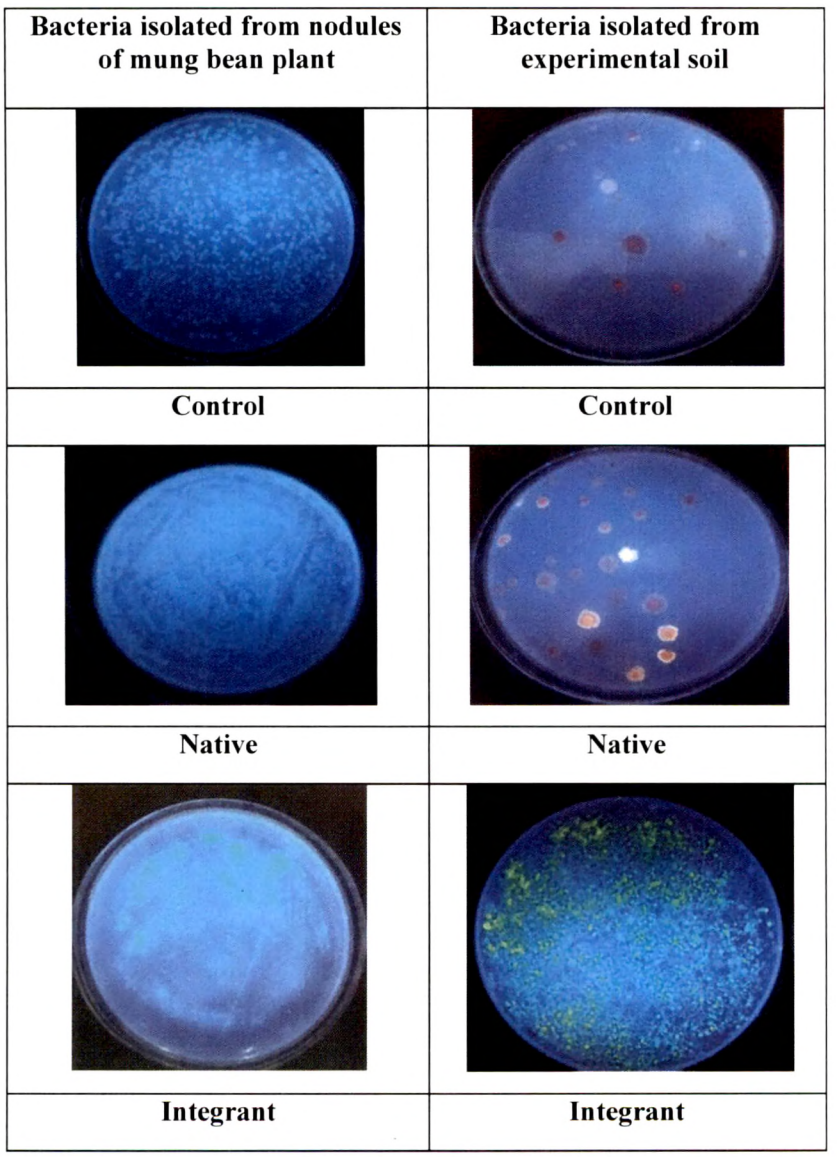


Fig. 8.4: Effect of genomic integrant on bacteria of rhizospheric soil and bacteroids of nodules.

Table 8.2: Effect of genomic integrant on number of *S. fredii* NGR 234 integrants in the rhizospheric soil and in nodules from 45 days old mung bean plants.

	Total bacterial count. CFU /mg of soil	Total bacterial count. CFU / nodule	Wt. of 10 nodules (mg)
Control	6×10^3	1.1×10^4	13.9
Native	1.6×10^4	1.7×10^4	14.2
Integrant	3.8×10^5	2.6×10^6	47.3

8.3.2 Effect *S. fredii* NGR 234 genomic integrant on nitrogenase activity, available Soil P and N, K content.

S. fredii NGR 234 genomic integrant increased ~5.5 fold and ~2.8 fold increase in nitrogenase activity of integrant and native, respectively, as compared to control (**Fig. 8.5**).

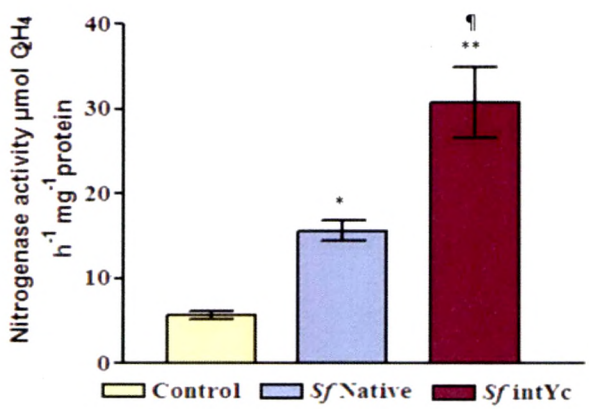


Fig. 8.5: Effect of *S. fredii NGR 234* genomic integrant on nitrogenase activity of mung bean at 45 Days after sowing. * Represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ** $P<0.01$ and *, ¶ $P<0.05$.

Inoculation with integrant (***Sf* intYc**) increased ~7.4 fold soluble P as compared to the uninoculated control and ~2.4 fold increase compared to native **(Table 8.3)**. The native strain resulted in ~3.1 fold increase in soil P while N and K levels remained unaltered compared to control. Also there was ~1.25 fold and ~2.32 fold increase in N and K content of the soil from plant inoculated with integrant compared to control.

Table 8.3: Effect of genomic integrant on the N, P and K content of rhizospheric soil from mung bean plants of 45 days old.

Soil	N Kg/hac	P_2O_5 kg/hac	K_2O kg/hac
Control	158.7	12.9	338.7
Native	161.6	40.0	364.9
***Sf* intYc**	198.9	95.7	788.9

8.3.3 Effect of genomic integrant on N P K content in plant and pods

Significant increase in N, P, K and protein content was observed in plants inoculated with the native (*S. fredii* NGR 234) and the integrant (***Sf* intYc).** N, K and protein showed ~1.5 fold increase in plants inoculated with the integrant compared to control and ~ 1.2 fold increase compared to native while P content increase was ~2.7 fold and ~1.64 fold, respectively, compared to control. Integrant also showed~1.64 fold increase in P and ~1.2 fold increase in N, K and protein content compared to native **(Table 8.4)**.

Significant increase in N, P, K and protein content was observed in pods from plants inoculated with the integrant (***Sf* intYc).** All parameters showed ~1.3 and ~1.1 fold increase in pods from plants inoculated with the integrant compared to control and native, respectively. Integrant also showed ~1.2 fold increase in N, P, K and Protein content compared to native strain (**Table 8.5**).

Table 8.4: Effect of genomic integrant on total plant N, P, K and protein content of 45 days old mung bean plants.

Bacterial inoculation	N %	P %	K %	Protein %
-	1.39	0.34	1.02	8.71
Native *Sf*	1.65	0.57	1.25	10.30
Sf intYc	2.07	0.93	1.57	12.96

Table 8.5: Effect of genomic integrant on N, P, K and protein content of pods from 45 days old mung bean plants.

Pods from plants with bacterial inoculation	N %	P %	K %	Protein %
-	2.61	1.40	1.05	16.29
Native *Sf*	3.05	1.51	1.24	19.08
Sf intYc	3.41	1.74	1.39	21.29

8.3.4 Growth parameters

8.3.4.1. Effect of *S. fredii* NGR 234 genomic integrant on growth parameters of mung bean plant.

S. fredii NGR 234 genomic integrant containing *E. coli* NADH insensitive *cs* and *S. typhimurium citC, vgb and egfp* gene cluster upon inoculation to mung bean resulted in significant increase in all growth parameters. In 20 day plants, ~1.3 fold increase in shoot length and ~2.8 fold increase in root length, ~2.4 fold increase in plant weight, ~2.9 and ~1.4 fold increase in leaf number and leaf area were found in comparison to control. Increase was also seen in all the above parameters in plants inoculated with genomic integrant inoculated with native *S. fredii* NGR 234 compared to control plants **(Fig. 8.5).**

In 45 day plants, ~1.45 fold increase in shoot length and ~1.35 fold increase in root length, ~2.5 fold increase in plant weight, ~2.3 and ~1.3 fold increase in leaf number and leaf area was seen (**Fig. 8.6**). In addition, there was ~1.7 fold and ~1.3 fold increase in chlorophyll content in the leaves of plants inoculated with integrant and native, respectively, compared to control **(Fig. 8.7).**

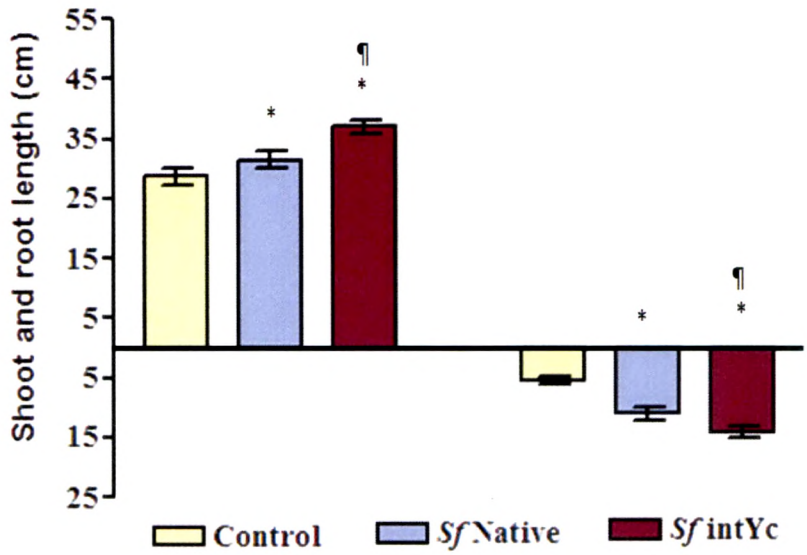


Fig. 8.5: Effect of *S. fredii* NGR 234 genomic integrant on shoot length and root length of mung bean at 20 Days after sowing. * represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. *,¶ $P<0.05$.

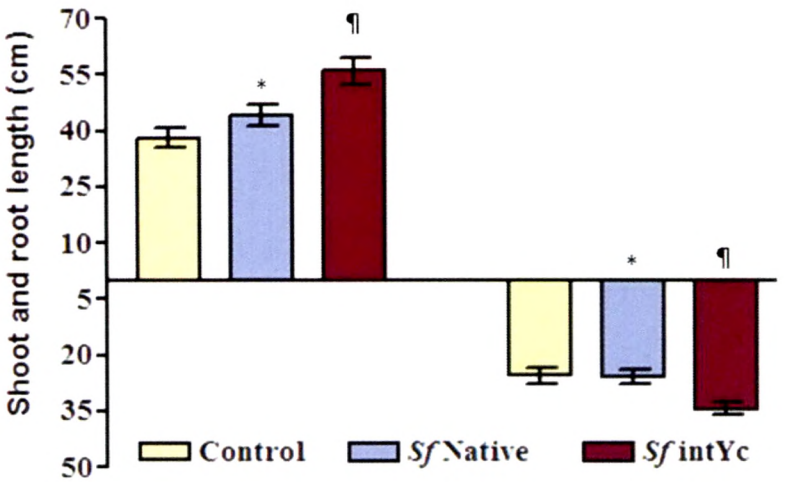


Fig. 8.6: Effect of *S. fredii* NGR 234 genomic integrant on shoot length and root length of mung bean 45 Days after sowing. * Represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. *,¶ P<0.05.

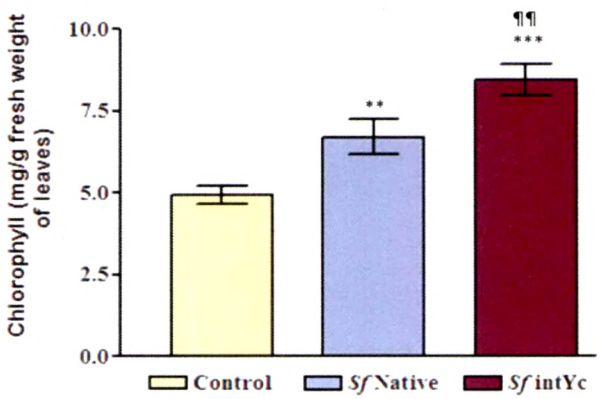


Fig. 8.7: Effect of *S. fredii NGR 234* genomic integrant on chlorophyll content of mung bean at 45 Days after sowing. * Represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ***, P<0.001, **, ¶ ¶ P<0.01.

There was ~2.4 fold, ~1.45 fold and ~1.6 fold increase in number, length and number of grains per pod, respectively, from plants treated with the integrant as compared to control. Fresh weight and dry weight of pods also showed ~2.2 and ~3.1 fold increase, respectively in integrant, compared to control.

Sf intYc genomic integrant in 20 days old plants showed, ~3.1 increase in number of nodules and ~4.3 fold increase in weight of 10 nodules compared to the control. Native Plants treated with native culture showed nodules having double weight compared to untreated plants and nodule number was increased by ~1.35 fold (**Table 8.6**). 45 days old plants treated with integrant showed less increase compared to increase seen in 20 days old plants (**Table 8.7**). ~2.8 increase in number of nodules and ~3.4 fold increase in weight of 10 nodules were found as compared to the control. Nodules from 45 days old integrant plants were ~3.3 times heavier than those of native plant.

Table 8.6: Effect of *S. fredii* NGR 234 genomic integrant on growth parameters of mung bean at 20 Days after sowing

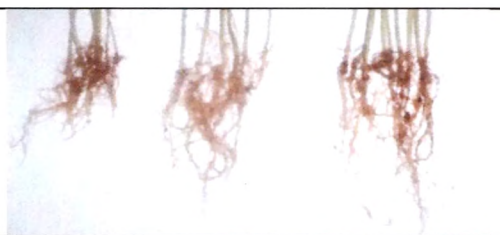
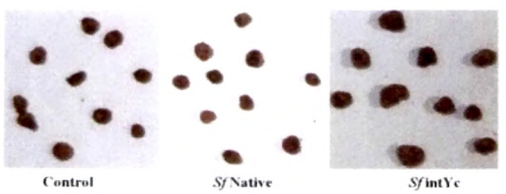
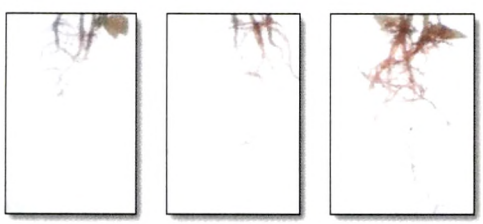
Details of 20 Days old Plants	Parameter	Control	Native	Integrant
	**Plant fresh weight in g**	2.20 ± 0.26	3.72 ± 0.10	5.33 ± 0.50
	No of leaves	6.33 ± 1.52	10.00 ±1.73	18.33 ±1.52
	Leaf area cm^2	17.33 ±2.08	21.66 ± 2.08	24.66 ± 2.51
	**No of nodules /plant**	23.33 ±3.51	31.66 ± 3.08	72.66 ± 2.51
	**Weight of 10 nodules in mg**	21.66 ± 2.08	46.00 ± 3.0	93.00 ± 4.58

Table 8.7: Effect of *S. fredii* NGR 234 genomic integrant on growth parameters of mung bean at 45 Days after sowing

Details of 45 Days old Plants	Parameter	Control	Native	Integrant
	**Fresh weight in g**	29.08 ± 5.22	44.93 ± 1.67	72.07 ± 13.33
	Dry weight in g	0.25 ± 0.13	0.33 ± 0.04	1.35 ± 0.06
	**No of leaves**	18.00 ± 3.00	23.00 ± 4.35	42.00 ± 5.29
	Leaf area in cm^2	26.33 ± 2.08	27.00 ± 1.00	34.66 ± 1.52

Contd...

Table 8.7: Effect of *S. fredii* NGR 234 genomic integrant on growth parameters of mung bean at 45 Days after sowing (contd.)

Details of 45 Days old Plants	Parameter	Control	Native	Integrant
	**No of pods/ plant**	10.33 ± 1.52	15.00 ± 3.00	24.66 ± 1.52
	Pod length in cm	6.26 ± 0.25	6.60 ± 0.26	9.13 ± 0.15
	**No of grains per/pod**	8.66 ± 1.5	10.00 ± 1.00	13.66 ± 1.52
	Pod fresh weight	1.176 ± 0.10	1.33 ± 0.15	2.58 ± 0.50
	Pods dry weight/mg	0.14 ± 0.04	0.25 ± 0.04	0.43 ± 0.11
	**No of nodules / plant**	12.33 ± 2.51	13.66 ± 2.05	34.33 ± 2.51
	**Weight of 10 nodules**	13.86 ± 1.95	14.20 ± 1.11	47.33 ± 3.05

The genomic integrant treatment had also decreased oxidative stress of mung bean plants as found in the specific activities of antioxidant enzymes (**Fig. 8.8**),

Superoxide Dismutase (SOD), **Catalase**, **Guaiacol Peroxidase** (POX) and **Ascorbate peroxidase** (APX), at 45 days after sowing. There was ~2.2 fold, ~1.4 fold, ~2 fold and ~ 1.5 fold decrease in POX, SOD, CAT and APX, respectively, in the plants inoculated with integrant compared to control. Compared to native, the integrants showed ~1.2 to ~1.4 fold decrease in the enzyme activities. PQQ levels increased by ~1.5 fold in the nodules and leaves of native as well as genome integrant inoculants as compared to control plant while (**Table 8.8**).

Table 8.8: PQQ levels in leaves and nodules of mung bean plants

Bacterial inoculation	PQQ ng/g fresh wt of nodule	PQQ ng/g fresh wt of leaves
-	3.26± 0.40	1.73± 0.25
S. fredii NGR 234	5.23± 0.51	2.43± 0.32
Sf intYc	6.10± 0.20	2.73± 0.32

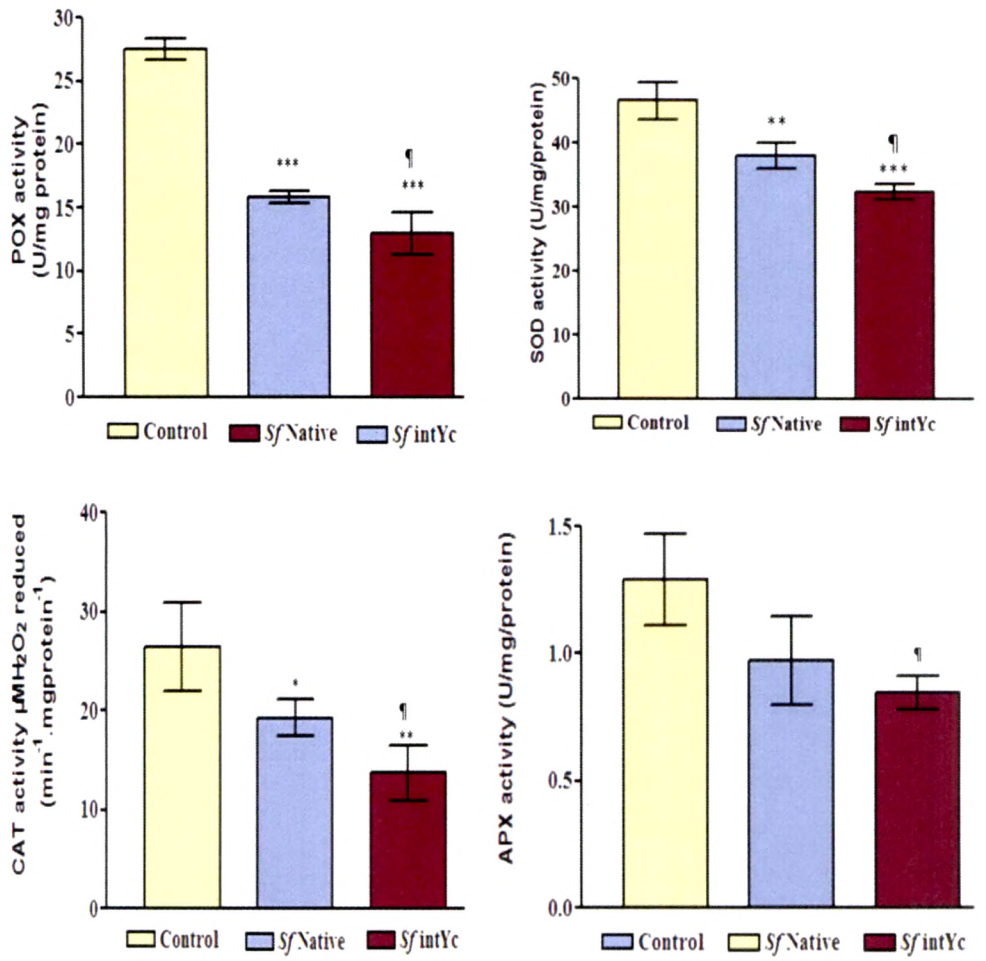


Fig. 8.8: Effect of *S. fredii NGR 234* genomic integrant on enzyme activities of mung bean at 45 Days after sowing. * represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ***, P<0.001, ** P<0.01 and *,¶ P<0.05 .

8.4 Discussion

Availability of phosphate in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes. Productivity of legumes is severely affected by P limitation, as both the plants and their symbiotic bacteria require P for nodule formation. Development of integrant of *S. fredii* NGR234 from TRP medium released 0.56 mM P as compared to 0.05 mM P of native strain. *S. fredii* NGR 234 integrant was efficient in increasing the N and P levels in soil.

Fluorescent colonies of *S. fredii* NGR234 containing integrant were present in rhizospheric soil and nodules isolated from plant (**Fig. 8.4**). Increase in cfu count of integrant was accompanied by increase in P, N and K content, respectively, as compared to native strain. Similar results of increase in soil P was found with co-inoculation of *Rhizobium* strain and PSM in chick pea and soybean (Argaw, 2011; Singh and Sharma, 2011). P levels play important role in nodule formation and nitrogen fixation as nitrogen fixation which is a high energy consuming process (Sulieman and Tran, 2012). Increase in free P content in soil helped plant growth and increased the number and weight of nodules compared to native strain.

Cfu count of *S. fredii* NGR 234 integrant in nodules also increased with concomitant increase in nitrogenase activity. Improvement in nodulation due to inoculation of P solubilizers was seen in chick pea and other leguminous plant (Tang et al., 2001). Enhanced nodulation after inoculation of the rhizobium strain increased available P for the plant as leguminous plant require high amount of P for nodule formation and maintenance of high rate of bacterial activity inside the nodule (Leidi et al., 2000; Zaman et al., 2007; Singh et al., 2011).

Increased nitrogenase activity led to increase in N, P, K and Protein content of whole plant in the integrant compared to control. Similar results were found with co

inoculation of legumes and crops with *Rhizobium* and different PSMs (Yazdani et al., 2011; Sharma et al., 2012; Tahir and Sarwar, 2013). Additionally, increase in NPK content of plant enhanced chlorophyll content and all growth parameters like fresh weight, dry weight, length of root, shoot and pod growth and yield parameters up to 100% compared to native. Simliar studies with barley and chick pea when grown in soil treated with insoluble phosphate and PSM *Mesorhizobium mediterraneum* PECA21 showed 100 and 125%, respectively, increase in the P content as compared to control (Peix et al., 2001).

P deficiency is one of the critical limiting factors, adversely affecting nodulation and N_2 fixation, and thus legume growth and productivity, worldwide (Tesfaye et al., 2007). *M. truncatula* plants inoculated with either the *S. meliloti* 102F51 or 2011 strain but due to P deficiency severely inhibited plant growth and development of nodules as well as N and P assimilation (Sulieman and Schulze, 2010*a*). Similar results were seen with co-inoculation studies with *Rhizobium*, PSMs, supplementation with fertilizers on *Zea mays*, cow pea, walnut, mung bean and chick pea (Gulati et al., 2010; Deepa et al., 2010; Yu et al., 2011; Jha et al., 2012; Verma et al., 2013).

S. fredii NGR 234 encodes *pqq* genes and incorporation of *yc* operon in the genome increased PQQ secretion probably due increased available P. PQQ is a strong antioxidant compared to ascorbate and other antioxidants. Addition of PQQ showed increased growth and scavenging of ROS and hydrogen peroxide (Choi et al., 2008; Ahmed and Shahab, 2010; Misra et al., 2012). Significant decrease in SOD, POX, CAT and APX activities after inoculation with the integrant of *S. fredii* NGR 234 indicates decreased oxidative stress in mung bean plants. 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 et al., 2000; Hemavathi et al., 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). 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 and 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 uninoculated stressed plants (Gururani et al 2012). Mung bean plant showed significant increase in all parameters related to growth and yield, decreased oxidative stress with *S. fredii* integrant compared to native. This clearly shows that *S. fredii* integrant is a strong nitrogen and phosphate solubilizer showing enhanced plant growth promoting ability. *In vitro* study of *vgb* on P solubilization, effect of inoculation of plasmid transformants on plant growth promotion and efficacy of integrant with respect to mung bean plant supplemented with super phosphate and urea will determine the potential of the genome integrants applicability in field conditions.