Chapter 6

Effect of *Sinorhizobium fredii* NGR 234 genomic integrant containing *pqq, gad, vgb* and *egfp* gene cluster on growth promotion of Mung bean plants.

6.1: Introduction

Legume crops are important as food, nutrition, and energy sources, accounting for over 30% of crop yields in the world (Graham and Vance, 2003). Most legumes form symbioses with soil nitrogen (N)-fixing bacteria (Lindström et al., 2010). The amount of organic N produced in legume-rhizobia symbiosis totals 20 to 22 million tons each year (Herridge et al., 2008). The average proportion of crop N derived from atmospheric N_2 is nearly 70% worldwide (http://faostat.fao.org/) considering this legume N_2 fixation is the most important N source in the agro ecosystems. Nitrogen contribution by legumes to other crops in the system depends on the legume species, biological N_2 fixation and the growth of the legumes, as determined by climate, soil and the management of residues. Grain legumes contribute less nitrogen than herbaceous legumes to subsequent crops in rotation, because most of the nitrogen fixed biologically by grain legumes is translocated to the grain and both the grain and the residues are constantly removed by humans and livestock (Rao and Mathuva, 1999). As legumes access atmospheric N_2 through the symbiotic relationship with rhizobia, they require minimal N fertilizer inputs (Van Kessel and Hartely, 2000). Nitrogen fixation by bacteria is costly to the legumes in terms of energy, still they are benefited considerably from this association when confined to soils that lack nitrogen compounds (Raven *et al.*, 2008; Taiz and Zeiger, 2010).

Nitrogen fixation by rhizobium not only helps its host legumes, but fixed N present in legume remnants helps to increase the soil fertility and thereby growth and yield of subsequent crops (Dakora, 2003). Inoculation of legumes with efficient strains of rhizobia has often resulted in significant increases in yields of various legume crops (Thies et al. 1991; Wani et al. 2007; Franche et al. 2009). The inoculation of seeds with *Rhizobium* increase 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; Togay et al., 2008). Inoculation of chickpea (*Cicer arietinum*) seeds with *R. ciceri* showed significant effect on seed yield, plant height, first pod height, number of pods per plant, number of seeds per plant, harvest index and 1,000 seed weight. But, nitrogen doses (applied at 0, 30, 60, 90, and 120 kg ha^{-1} level as

ammonium nitrate) had no significant effect on yield and yield components (Karasu et al., 2009).

The effect of phosphorus on BNF has been extensively studied as the effect is localized to specific soil, climate, legume variety and *rhizobial* strain (Plenchette and Morel, 1996; O'Hara, 2001; Hardarson and Atkins, 2003; Bowatte et al., 2006; Zaman-Allah et al., 2007). The number of nodules and plant growth of groundnut directly corresponded to plant available P (Lekberg and Koide, 2005). In a direct P rate response study using three varieties of cowpea (*Vigna unguiculata* cv. Amantin, It81D and Soronko), it was found that P application increased the size and number of nodules indicating the amount of BFN-N was increased (Ankomah et al., 1996). P addition significantly increased the total N in *Crotalaria micans* plants while the ratio of soil N uptake to BNF-N was not significant (Somado et al., 2006). These variable responses to P may be attributed to cultivar differences, including P uptake and assimilation (Sanginga, 2003).

The plant-growth-promoting rhizobacteria (PGPR) may induce plant growth promotion by direct or indirect modes of action (Lazarovits and Nowak 1997). On basis of activities they carry out, they are classified as as biofertilizers (increasing the availability of nutrients to plant), phytostimulators (plant growth promoting, usually by the production of phytohormones), rhizoremediators (degrading organic pollutants), and biopesticides (controlling diseases), mainly by the production of antibiotics and antifungal metabolites. Many PSMs along with phosphate solubilisation exhibit, 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). The beneficial effects of PGPR seen under greenhouse conditions are often not seen under field conditions and the results in terms of crop growth and yields are highly variable (Gyaneshwar et al., 2002; Martínez-Viveros et al., 2010). Environmental factors influence the level and reliability of PGPR in field condition (Dutta et al., 2010).

A large number of PGPR representing diverse genera have been isolated over past 50 years. Lately studies are done where strains having inherent plant growth promotion ability 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 (Bianco, 2006). Inoculation of *Medicago tranculata* plant with indole-3-acetic acid-overproducing mutant strain of *S. meliloti* improved both the shoot and root fresh weight, 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 them to survive, adapt and function better in the rhizosphere and improve plant nutrition.

Over-expression of genes involved in soil inorganic phosphate solubilization in natural PGPR can improve the capacity of microorganisms when used as inoculants (Vazquez et al., 2000). Studies carried out so far have shown that following appropriate regulations, genetically modified microorganisms can be applied safely in agriculture (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.

In addition to the nutrient availability and soil properties, oxygen limitation plays an important role in governing the MPS ability of bacterial transformants in field conditions (Ramírez et al., 1999). Oxygen is present in limited amounts in the rhizosphere which could limit the colonization and survival of rhizobacteria. Expression of *vgb* gene encoding VHb protein in heterologous hosts often enhances 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 increased upto ~56 mg/plant in bean plants (Ramirez et al., 1999). 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).

Present work deals with the effect of *vgb* and egfp gene integrated in *S. fredii* NGR234 along with *A. calcoaceticus pqq* cluster and *P. putida gad* operon (*Sf intPgvv*), *vgb* to improve the recombinant protein production and better survival of host under microaerobic conditions and *egfp* for bacterial tagging (Frey and Kallio, 2003).

6.1.2: Rationale of the Study

S. fredii NGR 234 can form nodule and carry out nitrogen fixation with 120 different species of legumes. *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 integrant, *Sf* intPgv, 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 is to determinate the effects of *Sf* intPgv on plant height and weight, leaf area, chlorophyll content, number and size of pods, and grain yield and different enzyme activities on mung bean plant.

not clear! you did not present/ discuss results with B.J & M.L.

6.2 Experimental design

6.2.1: Bacterial strains used in this study

Table 6.1: Bacterial strains used in this study.

Plasmid/Strains	Characteristics	Source or Reference
Sinorhizobium fredii NGR 234	Accession number NC_012587.1	NCBI
Sf intPgv	Genomic integrant of *S. fredii* containing *lac-pqq-gad, vgb, egfp* Ap^r	This study Chapter 6

6.2.2 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 *pqq-gad* operon genomic integrant (Int). In the experiment, one control was used, where no inoculum was added and designated as control (C).

was a 'prod of Concept' first performed in sterile sand microcosms to demonstrate the superiority of the genetically modified strain ?

6.2.3 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).

6.2.4 Growth parameter assessment

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

6.2.5 Biochemical characterization

SOD, Catalase, Ascorbate Peroxidase, Guaicol Peroxidase and Nitrogenase enzyme activities were estimated at 45 days after sowing (DAS). Experimental details for the preparation of sample and enzyme assays have been given in Section .....

6.2.6 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 the with standard PQQ on spectrofluorometer. Fluorescence was monitored at *ex* 360 and *em* 480 nm.

6.2.7 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.

6.2.8 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.

6.3 RESULTS

6.3.1 Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv, on bacteria of rhizospheric soil and bacteroids of mung bean nodules.

Fluorescent colonies under UV light were only seen in the rhizospheric soil and nodules of mung bean plants inoculated with *S. fredii* NGR234 integrant, *Sf* intPgv (**Fig.6.1**). Mucoid colonies of *Rhizobium* were seen in the control and native plates while fluorescent colonies of *S. fredii* NGR234 with the integrant were seen in the plate from nodules from the inoculated mung bean plant. There was increase by 26 fold and 160 fold in CFU count from the rhizospheric soil and bacteroids from nodules of mung bean plant, respectively, inoculated with the integrant (**Table 6.2**).

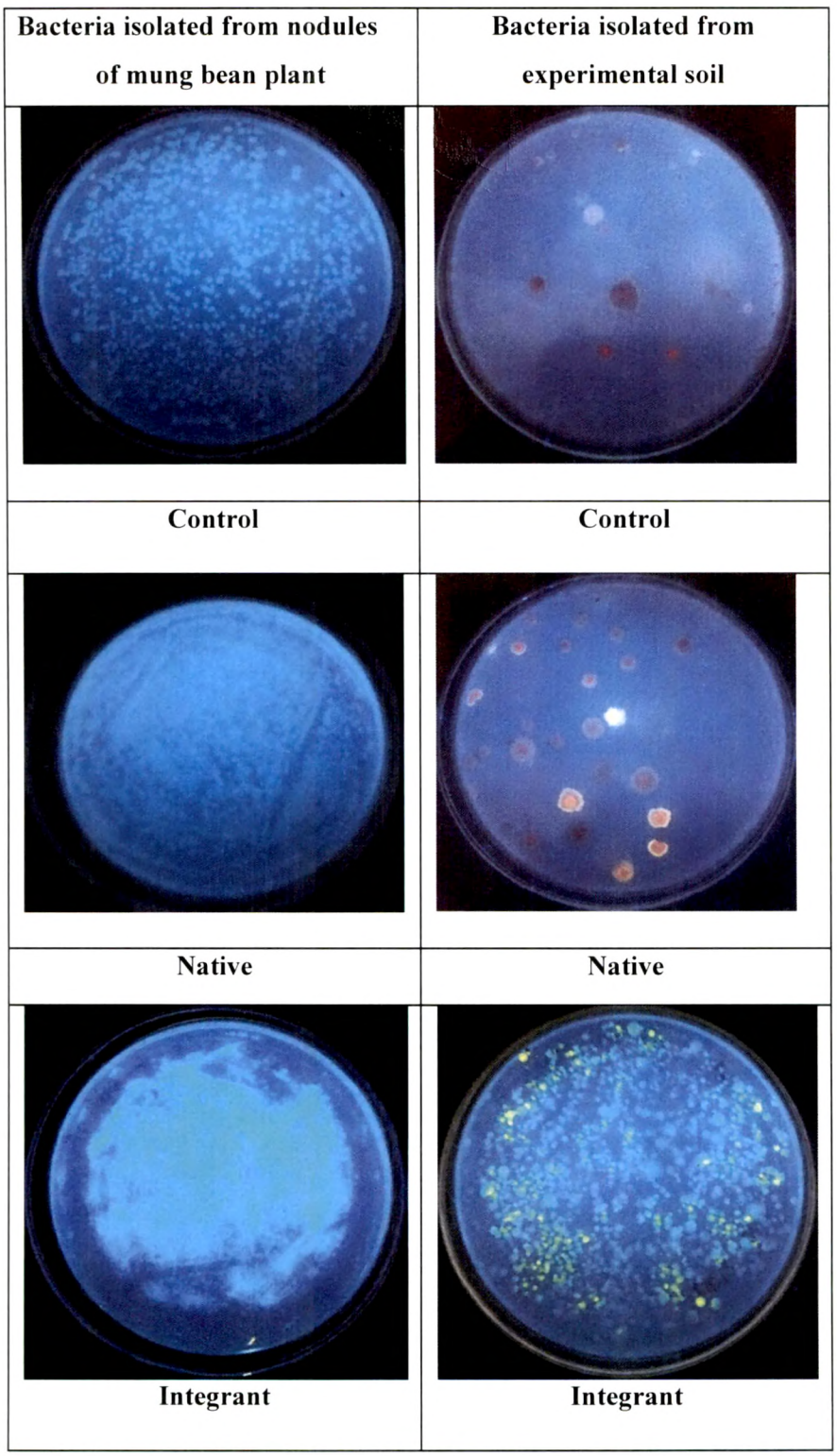


Fig. 6.1: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv, on bacteria of rhizospheric soil and bacteroids of nodules.

Table 6.2: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv 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	1.7×10^6	14.2
Integrant	4.2×10^5 (2.6)	2.7×10^8 (1.6)	46.3

6.3.2: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv on nitrogenase activity, available soil P, N, and K content.

There was a ~5.4 fold and ~2.8 fold increase in nitrogenase activity of integrant and native, respectively, compared to control. Compared to native, integrant inoculation showed ~2 fold increase in activity (**Fig. 6.2**).

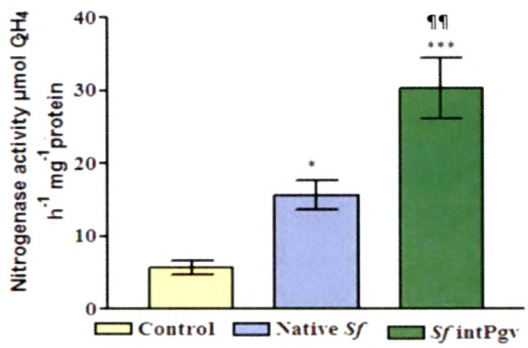


Fig. 6.2: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv on nitrogenase activity of mung bean at 45 Days after sowing. * represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ** $P<0.01$ and *,¶ $P<0.05$.

Inoculation with *Sf* intPgv integrant increased ~7.9 fold soluble P as compared to the uninoculated control and ~2.5 fold increase compared to native (**Table 6.3**). The native strain inoculation resulted in ~3.1 fold increase in soil P while N and K levels remained unaltered compared to control. Also there was ~1.28 fold and ~3.53

fold increase in N and K content of the soil from plant inoculated with integrant compared to control.

Table 6.3: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv, 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	156.7	12.9	336.7
Native	161.6	40.0 3.1	364.9
***Sf* intPgv**	203.5	101.4	1197.5

6.3.3 Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv 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* NGR234) and the integrant (*Sf* intPgv) (**Table 6.4**). 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 ~3.23 fold and ~1.64 fold, respectively, compared to control. Integrant also showed ~1.92 fold increase in P and ~1.2 fold increase in N, K and Protein content compared to native strain.

Significant increase in N, P, K and protein content was observed in pods from plants inoculated with the integrant (*Sf* intPgv) **(Table 6.5).** All parameters showed ~1.3 fold increase in pods from plants inoculated with the integrant compared to control and ~1.2 fold increase in pods compared to native.

Table 6.4: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv, 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	6.71
Native	1.65	0.57	1.25	10.30
***Sf* intPgv**	2.14	1.10	1.58	13.38

Table 6.5: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv , 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 intPgv	3.48	1.89	1.36	21.76

6.3.4 Growth parameters

6.3.4.1. Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv on growth parameters of mung bean plant.

Sf intPgv showed significant increase in all the growth parameters. 20 day plants showed ~1.2 fold increase in shoot length and ~2.6 fold increase in root length, ~2.3 fold increase in plant weight, ~2.8 and ~1.5 fold increase in leaf number and leaf area **(Table 6.6; Fig. 6.3)**. Increase was also seen in all the above parameters in plants inoculated with genomic integrant compared to plants inoculated with native *S. fredii*.NGR234.

In 45 days plant, ~1.5 fold increase in shoot length and ~1.4 fold increase in root length, ~2.6 fold increase in plant weight, ~2.2 and ~1.36 fold increase in leaf number and leaf area was seen (**Fig. 6.4**). In addition, there was ~1.8 fold and ~1.4 fold increase in chlorophyll content in the leaves of plants inoculated with integrant and native, respectively, compared to control (**Fig. 6.5**).

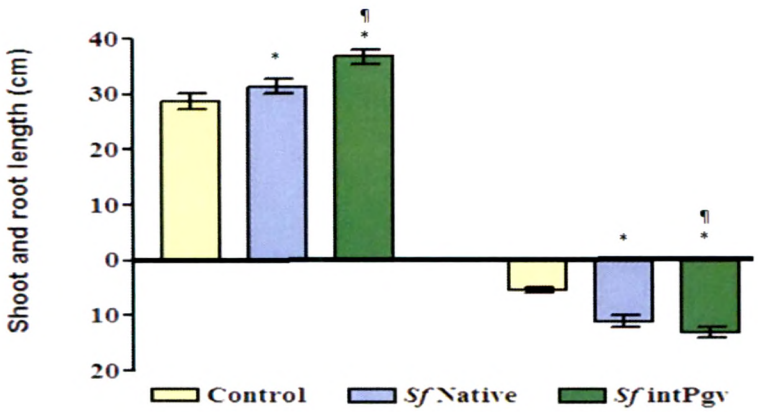


Fig. 6.3: Effect of *S. fredii* genomic integrant on shoot length and root length of mung bean at 20 Days after sowing.

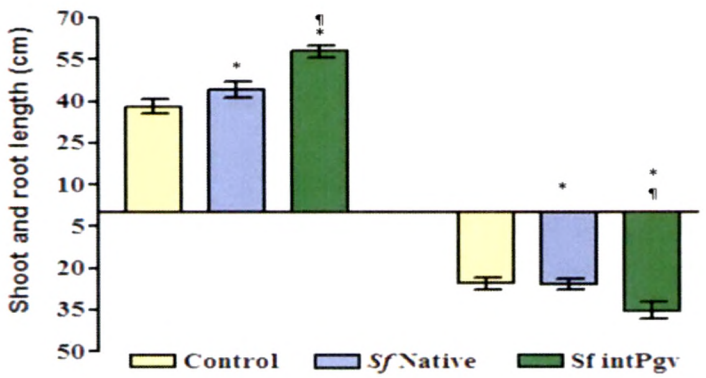


Fig.6.4: Effect of *S. fredii* genomic integrant on shoot length and root length of mung bean at 45 Days after sowing.

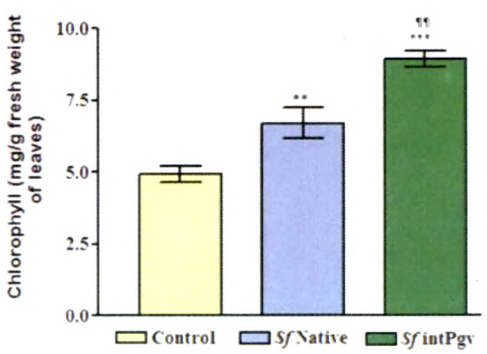


Fig. 6.5: Effect of *S. fredii NGR 234* genomic integrant on chlorophyll content of mung bean at 45 Days after sowing. * Represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ***, P<0.001, **, ¶ ¶ P<0.01.

6.3.4.2 Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv on Pod formation.

There was ~2.4 fold, ~1.47 fold and ~1.6 fold increase in number, length and number of grains per pod, respectively from plants treated with integrant, compared to control. Fresh weight and dry weight of pods also showed ~2.32 and ~3.1 fold increase, respectively in integrant, compared to control.

Sf intPgv genomic integrant in 20 days old plants showed, ~3.17 increase in number of nodules and ~4.9 fold increase in weight of 10 nodules compared to the control. Plants treated with native culture showed nodules having double weight compared to untreated plants and nodule number was increased by ~1.35 fold. (**Table. 6.6**).

45 days old plants treated with integrant showed less increase compared to increase seen in 20 days old plants. There was ~2.8 increase in number of nodules and ~3.34 fold increase in weight of 10 nodules compared to the control. Nodules from 45 days old integrant plants were ~3.3 times heavier than those of native plant. (**Table. 6.7 and Table. 6.8**).

Table 6.6: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv , on growth parameters of mung bean at 20 Days after sowing.

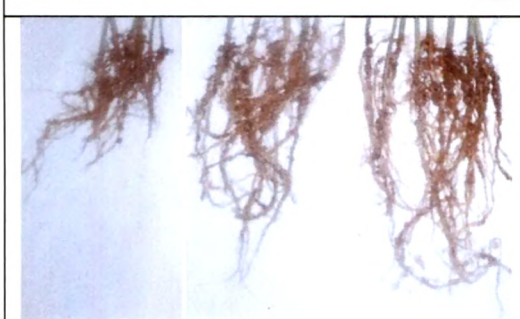
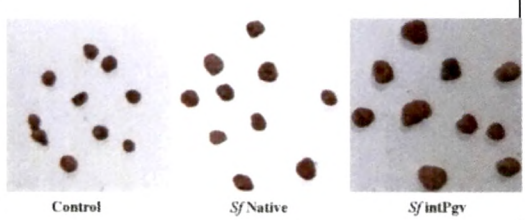
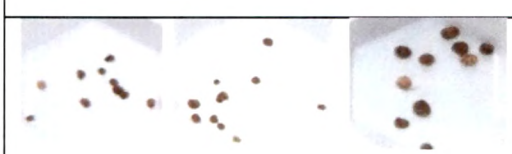
Details of 20 Days old Plants	Parameter	Control	Native	Integrant
	Plant fresh weight in gm	2.20 ± 0.26	3.72 ± 0.10	5.23± 0.51
	No of leaves	6.33 ± 1.52	10.00 ±1.73	17.66± 1. 52
	Leaf area cm^2	17.33 ±2.08	21.66 ± 2.08	25.66 ± 1.5
	No of nodules /plant	23.33 ±3.51	31.66 ± 3.08	74.00 ±2.00
	Weight of 10 nodules in mg	21.66 ± 2.08	46.00 ± 3.0	106.33± 4.04

Table 6.7: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv 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	75.43 ±7.51
	Dry weight in g	0.25 ± 0.13	0.33 ± 0.04	1.40 ±0.20
	No of leaves	18.00 ± 3.00	23.00 ± 4.35	39.66 ±6.80
	Leaf area in cm^2	26.33 ± 2.08	27.00 ± 1.00	36.00 ±2.00

Contd...

Table 6.8: 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 ±3.05
	Pod length in cm	6.26 ± 0.25	6.60 ± 0.26	9.20 ±0.19
	No of grains per/pod	8.66 ± 1.5	10.00 ± 1.00	14.00 ±2.00
	Pod fresh weight	1.176 ± 0.10	1.33 ± 0.15	2.32 ±0.05
	Pods dry weight/mg	0.14 ± 0.04	0.25 ± 0.04	0.45 ±0.05
	No of nodules / plant	12.33 ± 2.51	13.66 ± 2.05	34.66 ±4.16
	Weight of 10 nodules	13.86 ± 1.95	14.20 ± 1.11	46.33± 1.52

6.3.6. Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv on PQQ secretion.

Table 6.9: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv, on PQQ secretion from leaves and nodule of 45 days old mung bean plants.

Bacterial inoculation	PQQ (Leaves)	PQQ (Nodules)
-	1.73±0.25	3.26± 0.40
Native	2.43±0.32	5.23± 0.51
Sf intPgv	35.33±4.04	86.10± 0.20

6.3.5. Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv antoxidant enzyme activities.

To determine the effect of *S. fredii* 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 sowing. All these enzymes showed significant decrease in the integrants as well as in the plants inoculated with native culture compared to the control (**Fig. 6.9**). There was ~3.8 fold, ~2.0 fold, ~2.75 fold and ~ 2.6 fold decrease in POX, SOD, CAT and APX, respectively, in the plants inoculated with integrant compared to control. Significant decrease in all the antioxidant enzymes was also seen in the native plant compared to control. Compared to native, the integrants showed ~2 fold decrease in the enzyme activities.

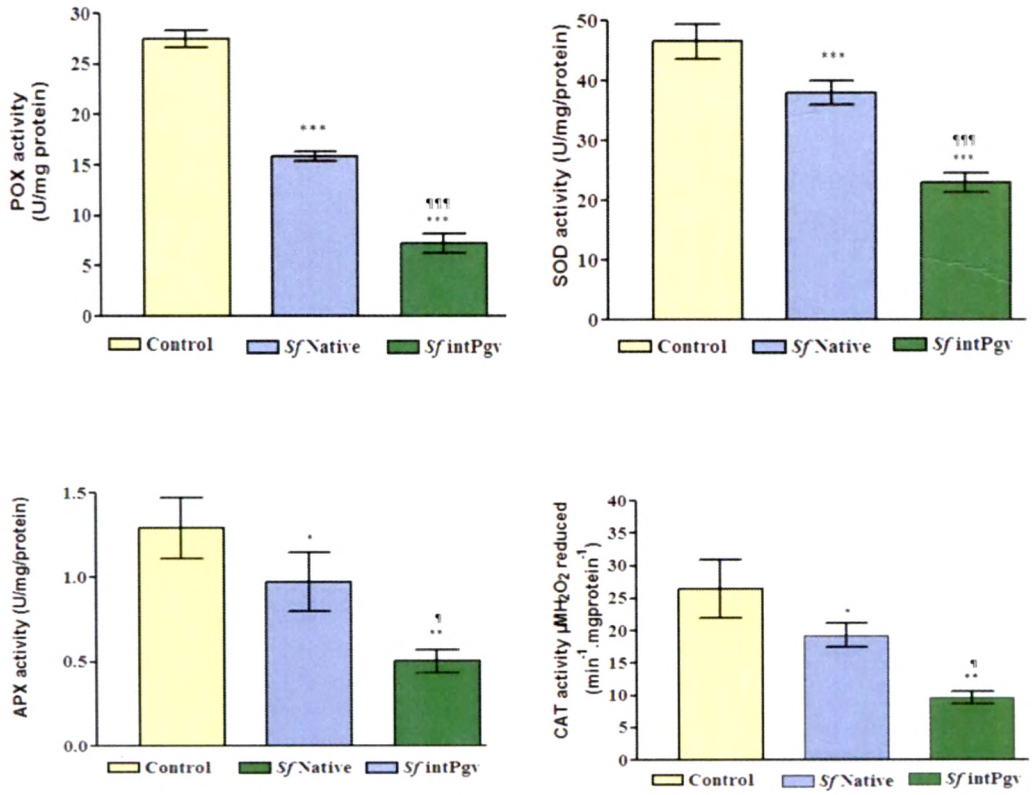


Fig. 6.9: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv on enzyme activities of mung bean at 45 Days after sowing. * represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ***, P<0.001, ** P<0.01 and *,¶ P<0.05 .

Discussion

Mung bean [*Vigna radiata* (L.) Wilczek], is one of the important and well-known economic crops extensively cultivated in Asia during warm season. Soil bacteria *rhizobia* generally associated with legumes (Appunu et al., 2009; Melchiorre et al., 2010). N fixation requires greater P requirement (Israel, 1987). Productivity of legumes are severely affected by P limitation, as both the plants and their symbiotic bacteria require P for nodule formation. Symbiotic effectiveness of rhizobial inoculants for a wide variety of legumes can be improved by co-inoculation with suitable non-rhizobial plant growth promoting bacteria (PGPB) (Lazdunski et al., 2004). Release of organic acids and enzymes by PSMs converts unavailable P to readily available form which can be taken by plants. Co-inoculation of PSMs with crop specific rhizobia improves root infection which results in better nodulation and grain yield. Co-inoculation of *Pseudomonas* with rhizobia enhance nodulation, nitrogen fixation, plant biomass and grain yield in various leguminous crops such as alfalfa, pea, soybean, green gram and chickpea (Mishra et al., 2009). Co-inoculation of rhizobia with *Bacillus*, specifically *Bacillus thuringiensis*, *Bacillus megatrium* and *Bacillus cereus* significantly promotes nodulation, plant growth and grain yield (Halverson and Handelsman, 1991; Mishra et al., 2009). Co-inoculation of arbuscular mycorrhizae with *Bradyrhizobium* sp. proved to be very helpful in improving mung bean growth (Yasmeen et al., 2012a, b).

Rhizobium species are host specific in nodule formation, *S. fredii* NGR234 can form nodule formation with 120 species, considering this attribute of *S. fredii* we carried out studies to determine the effect of *S. fredii* integrant on growth and yield of mung bean (Pueppke et al., 1999).

Presence of PQQ and GADH in *S. fredii* NGR234 genomic integrant, *Sf* intPgv, resulted into increased GDH and GADH activity which resulted into release of high amount of GA and 2-KGA on TRP medium. Although *S. fredii* native strain contains apoGDH, it does not secrete these organic acids as it lacks complete set of genes required for PQQ biosynthesis. *S. fredii* integrant was efficient in releasing P from RP which could account for the increased number of *S. fredii* integrant in the rhizospheric soil. Secretion of GA and 2-KGA by this integrant could increase in P, and K content of the soil as organic acids are known to also solubilize K minerals

(Barker et al., 1998). More number of genome integrants in the rhizosphere coupled with increased EPS and biofilm formation may have facilitated better infection and nodule formation leading to the increased number of nodule and bacteroids per nodule. Similar results of increased number of nodules were also found with coinoculation of *Rhizobium* strain and PSM in chick pea and soybean (Argaw, 2011; Singh and Sharma, 2011). Additionally, inoculation of genome integrant increased the P and K status of plants which may have played an important role in nodule formation and nitrogen fixation as nitrogen fixation is high energy consuming process, 16 ATP molecules to fix one molecule of N_2.

Enhanced nitrogenase activity observed is associated with the increase in number of nodules and number of bacteroids per nodule in plants inoculated with *S. fredii* NGR234 genomic integrant, *Sf* intPgv. 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 suggested an increase in 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. 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 resulted into increase in chlorophyll content and all growth parameters like fresh weight, dry weight, length of root, shoot and pod. 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). 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, chick pea

(Gulati et al., 2010; Deepa et al., 2010; Yu et al., 2011; Jha et al., 2012, Verma et al., 2013).

ApoGDH of *S. fredii* NGR234 genomic integrant, *Sf* intPgv, is reconstituted into active GDH. *S. meliloti gcd* was shown to be expressed at very early stages of plant/bacteria interactions at the rhizosphere level, and was maintained throughout subsequent symbiotic stages, while *gcd* mutant of *S. meliloti* showed delay in nodule emergence and a reduced ability for nodulation (Bernardelli et al., 2008). Similarly, Myo-inositol mutants of *S. fredii* and *R. leguminosarum* showed diminished nodulation competitiveness with the wildtype (Fry et al. 2001; Jiang et al. 2001), while mutants of *R. leguminosarum* unable to catabolize rhamnose showed altered competitive behaviour (Oresnik et al. 1998). Bernardelli and his co-workers speculated that GDH mutant showed decreased EPS production, which plays a prominent role in first step of root colonization for nodule formation. Thus, higher GDH activity of genome integrant could have contributed to improved infection and nodule numbers in mung bean plant.

Plants have different enzymes and mechanisms to decrease the concentration and deleterious effect of ROS species (Taiz and Zeiger, 2010). Presence of *A. calcoaceticus pqq* gene cluster is integrated in genome of *S. fredii* could release PQQ in nodules which in turn may have been transported to leaves of *S. fredii* inoculated plants. This is supported by the fact that Arabidopsis, tobacco and cucumber plants inoculated with *P. fluorescens* B16 showed the presence of micromolar levels of PQQ in leaves which contribute towards growth while *pqq* mutant bacterial strain inoculation did not have PQQ (Choi et al., 2008). Significant decrease in SOD, POX, CAT and APX activities integrant inoculated plants in compared to the native inoculant plants could be attributed to the strong antioxidant activity of PQQ which is better than that of ascorbate and other antioxidants (Rucker et al, 2009; Misra et al., 2012). PQQ is known increase growth and scavenging of ROS and hydrogen peroxide (Choi et al., 2008; Ahmed and Shahab, 2010; Misra et al., 2012). Inoculation of PGPR reported to reduce oxidative stress in plants (Sgherri et al., 2000; Upadhyaya et al., 2010). Increased PQQ levels could also help the plants to tolerate abiotic stress conditions as cause increase in ROS formation such as superoxide radical (O_2^-), hydrogen peroxide, and hydroxyl radicals (OH) increase. 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 unninoculated stressed plants (Gururani et al 2012). Thus, *S. fredii* NGR234 genomic integrant, *Sf* intPgv, may also be helpful in alleviating abiotic stresses in plants. Thus, present study demonstrates that *S. fredii* integrant, is efficient nitrogen fixer and phosphate solubilizer, and contributes significantly for plant growth. *S. fredii* NGR234 has a broad host specificity, thus, the genomic integrant, *Sf* intPgv, could also be effective in many legumes. However, further studies on determining the effect of *vgb* gene on growth, comparing the plant growth promoting ability of plasmid transformants *vs* genomic integrant *vs* chemical N and P fertilizers are necessary for their potential benefit in agriculture.

Summary

Rhizobium species are well known for its nitrogen fixing ability in legumes through nodule formation. There is interplay of many factors responsible in nodule formation and symbiotic nitrogen fixation contributed by specific legume host and its symbiont *Rhizobium* species (Ref). one of it being available levels of phosphate in rhizosphere. Phosphate solubilizing microorganisms in rhizosphere increases levels of phosphorus by secreting organic acids like gluconic acid, citric acid, oxalic acid etc. In Gram Negative bacteria like *Pseudomonas* and *Rhizobium* carbohydrate is metabolized through direct oxidation and ED pathway as EMP pathway is absent. In *Rhizobium* species, gluconic acid synthesis is mediated by periplasmic PQQ dependant glucose dehydrogenase enzyme. *Mesorhizobium loti* and *Rhizobium leguminosarum* show the presence of apoenzyme form of glucose dehydrogenase, but lack certain genes or whole cluster of *pqq* operon, while both genes are present in *Bradyrhizobium japonicum, Ensifer meliloti* and *Sinorhizobium fredii* NGR234.

Two plasmids containing 1.3 kb *E. herbicola pqqE* (pJNK1) and 5.1 kb *pqq* gene cluster of *Acinetobacter calcoaceticus* (pJNK5) were constructed in pUCPM18Gm^r under *Plac* promoter and transformed in *B. japonicum (Bj), M. loti (Ml)* and *S. fredii* NGR 234 *(Sf)*. *Bj* (pJNK1) and *Ml* (pJNK1) secreted ~0.315 μM and ~0.159 μM PQQ in medium, while *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) secreted ~7.3 μM, ~7.0 μM and ~8.75 μM PQQ in medium. GDH activity was found to be ~18 U and ~19 U in *Bj* (pJNK1) and *Ml* (pJNK1), respectively, while in *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) it was ~177 U, ~ 144 U and ~210 U, respectively. *Bj* (pJNK1) and *Ml* (pJNK1) did not show significant phenotype on Pikovskaya and TRP plate, while *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) showed significant phenotype on both the plates. *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) secreted ~27mM, ~28 mM and ~29 mM of gluconic acid, respectively. Under aerobic condition, *Bj* (pJNK1) and *Ml* (pJNK1) solubilized ~60 μM and ~ 64 μM P on 100 mM Tris-Cl pH 8.0 buffered rock phosphate medium containing 50 mM glucose, while *Bj* (pJNK5), *Ml* (pJNK5) and *Sf* (pJNK5) solubilized ~0.253 mM, ~0.213 mM and ~ 0.310 mM P, respectively. Thus, incorporation of *pqq* gene cluster, and

not *pqqE,* confers mineral phosphate solubilization ability in *Rhizobium* transformants by solubilizing rock phosphate even under buffered medium.

Gluconic acid (GA) is produced via direct oxidation pathway by membrane bound pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GDH). Apo protein of PQQ-GDH is found to be present in *Rhizobium* species, which oxidizes glucose to GA in periplasmic space. Genetic modification of apoGDH containing *Bradyrhizobium japonicum, Mesorhizobium loti* and *Sinorhizobium fredii* NGR234 strains with *Acinetobacter calcoaceticus pqq* gene cluster secreted ~ 28 mM GA and released P when grown on 50mM Glucose TRP medium. GA can be further oxidized to 2-ketogluconic acid (2-KGA) in the periplasm by FAD-dependent gluconate dehydrogenase (GADH), encoded by *gad* operon. 2-KGA (pKa 2.6) is much stronger than GA (pKa 3.86) and efficiently chelates calcium in soil and helps in mineral phosphate solubilisation. Many bacteria are known to secrete 2KGA.

Genome sequences indicated that *B. japonicum* and *M. loti* lack *gad* genes and putative genes are present in *S. fredii* NGR234. Plasmid harboring 5.1 kb *pqq* gene cluster of *A. calcoaceticus* along with 3.8 kb *P. putida* KT 2440 *gad* operon were constructed in pUCPM18Gm^r under *Plac* promoter (pJNK6) and transformed in *B. japonicum, M. loti* and *S. fredii* NGR234 strains. GADH activity in *Bj* (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) was found to be 340.9 U, 438.4 U and 326.3 U, respectively, while GDH activity was 182.8 U, 151.2 U and 219.0 U, respectively. *Bj* (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) secreted 21 mM, 20 mM and 18 mM of GA, respectively, along with 14 mM, 13.7 mM and 15 mM of 2-KGA. Transformants secreted 7 µM, 7 µM and 9 µM PQQ in medium and also released 0.642 mM, 0.534 mM and 0.609 mM P on TRP minimal medium containing 50 mM glucose. Thus, heterologus overexpression of *gadh* gene and *pqq* gene cluster (pJNK6) together significantly enhanced the MPS efficacy of *Rhizobium* strains.

Co-inoculation of *Rhizobium* strains with PSMs have led to remarkable increase in nitrogen fixation, plant growth and yield. Genetic modification of *Rhizobium* species is

largely carried out to enhance, its nitrogen fixation ability, nodule occupancy, multiple host infection, withstand biotic - abiotic stress and biocontrol activity. Our studies show that genetic modification by heterologus overexpression of *A. calcoaceticus pqq* gene cluster along with *Pseudomonas putida gad* operon in *B. japonicum*, *M. loti* and *S. fredii* NGR234 expressed in pUCPM18 Gm^r plasmid under *Plac* promoter secreted,~ 20 mM GA and ~15 mM 2KGA , ~32 fold increase in 2KGA and P release compared to pJNK5 transformants was observed. Higher 2KGA secretion increased the MPS efficiency of *Rhizobium* transformants. Heterologous overexpression of genes using plasmids in Rhizobia known to affect growth, organic acid secretion, loss of genes, nodulation and nodule occupancy. Our present study is to achieve stable chromosomal integration of *Vitreoscilla hemoglobin* (*vgb*), *egfp, A. calcoaceticus pqq* gene cluster along with *Pseudomonas putida gad* operon in *B. japonicum*, *M. loti* and *S. fredii* NGR234 using Tn7 based integration system at *att* site and check its growth and MPS abilities. GADH activity in *Bj* intPgv, *Ml* intPgv and *Sf* intPgv was found to be 193.06 U 200.10 U and 162.9 U, respectively, while GDH activity was 129.83 U, 80.23 U and 147.03 U, respectively. *Bj* intPgv, *Ml* intPgv and *Sf* intPgv secreted 20.69 mM, 18.40 mM and 22.41 mM of GA, respectively, along with 7.87 mM, 6.73 mM and 8.6 mM of 2-KGA. Transformants secreted 3.4 µM, 3.64 µM and 4.26 µM PQQ in medium and also released 0.406 mM, 0.394 mM and 0.393 mM P. Integrant showed MPS ability on 50 mM Tris-Cl RP minimal medium containing 50 mM glucose, thus there is decrease in MPS ability by integrant compared to overexpression through plasmid.

Symbiotic effectiveness of rhizobial inoculants for a wide variety of legumes can be improved by co-inoculation with appropriate plant growth promoting bacteria).In this study, mung bean plants were inoculated with *S. fredii* NGR234 genomic integrant, *Sf* intPgv. Presence of PQQ and GADH in *Sf* intPgv resulted into increased GDH and GADH activity which resulted into release of high amount of GA and 2-KGA on TRP medium. Secretion of GA and 2-KGA by the plant inoculated with the integrant showed ~7.9,~3.5 and ~1.3 fold increase in P, K and N, respectively, from plant inoculated with integrant compared to the untreated plant . Availabilty of these micronutrients facilitated better infection and nodule formation leading to the increased number of nodule and

nutrients

bacteroids per nodule. Better root colonization was evident by increase in nitrogenase activity. Significant (~5.4 fold) enhancement in nitrogenase activity compared to the control plant, observed is associated with ~2.5 and ~3.3 fold increase in number and weight of nodules, respectively in plants inoculated with *S. fredii* NGR234 genomic integrant, *Sf* intPgv. Number, length, weight of pods and number of grains per pod showed ~2.0 to ~2.5 fold increase in the integrant treated plant, compared to control plant. Increase in PQQ levels by ~---fold by the presence of PQQ in the integrant helped in increase in activity of anti oxidant enzymes by ~2 to ~4 fold compared to control. This suggests decrease in abiotic stress of the plant. Thus, present study demonstrates that *S. fredii* integrant, is efficient nitrogen fixer and phosphate solubilizer, and contributes significantly for plant growth. *S. fredii* NGR234 has a broad host specificity, thus, the genomic integrant, *Sf* intPgv, could also be effective in many legumes.