Genetic manipulation of carbohydrate catabolism in *Rhizobium* spp. for mineral phosphate solubilization

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STATEMENT UNDER O. Ph.D. 8/(iii) OF

THE M. S. UNIVERSITY OF BARODA, VADODARA

This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Dr. G. Naresh Kumar.**

Sonal Shah

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Date: 16/9/13

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph. D. degree of The M. S. University of Baroda by Ms. Sonal Shah is her original work. The entire research work and the thesis have been built up under my supervision.

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DEDICATED

TO

ATMIYA

SAMAJ

Expression of Gratitude :

I am overwhelmed writing the most beautiful and the most honorable chapter of this long journey. This journey would not have reached its ultimate destination without this chapter.

Have used lot of mathematics and statistics during work, applying same for this journey , became aware that for every step there were ± 2 with me for each step taken forward. I would therefore like to express my indebtedness to every individual who has been a crucial part of this endeavour.

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Well.. a small child was told in a class to write 5 lines on mother, small child when asked why is he not writing anything?, he replied " 26 alphabets are not enough to express my mother, so how can I write 5 lines about her using those 26 alphabets". I am facing the same dilemma when I think how to offer my heartfelt gratitude to my research Supervisor, ***Prof. G. Naresh Kumar*** *for accepting me as his student. Sir, I have learned a lot from you, you taught me to laugh ,to believe, to see beyond the curtain, to think, to understand and solve problems.. list is endless..but the most important take home message I got every time I had been with you is " God has made everything simple, we human beings make it complicated" . Sir, you taught me how to*

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You blessed me with your service...

You are the doer; I am merely your instrument.

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List of contents

List of Abbreviations

6-PG	6-phosphogluconate
Ap	Ampicillin
ATP	Adenosine triphosphate
bp	base pair (s)
cAMP	Cyclic adenosine monophosphate
CS	Citrate synthase
CTAB	Cetyl trimethyl ammonium bromide
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DTT	1, 4-Dithiothreitol
ED /EDD	Entner-Doudoroff Pathway
EDA	KDPG Aldolase
EDTA	Ethylene diamine tetra acetic acid
EMP	Embden–Meyerhof–Parnas pathway
ETC	Electron transport chain
G-6-P	Glucose-6-phosphate
G-6-PDH	Glucose-6-phosphate dehydrogenase
GADH	Gluconate dehydrogenase
GDH	Glucose dehydrogenase
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
ICDH	Isocitrate dehydrogenase
ICL	Isocitrate lyase
kb	kilobase (s)
kDa	Kilodalton
KDPG	2-keto-3-deoxy-6-phosphogluconate
Km	Kanamycin
LDH	Lactate dehydrogenase
LB	Luria broth
MDH	Malate dehydrogenase
ME	Malic enzyme
mM/M	Millimolar/Molar
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
ng	Nanogram
nm	Nanometer
OAA	Oxaloacetate
OD	Optical Density
Ori	Origin
P/Pi	Phosphate / inorganic phosphate
PCR	Polymerase Chain Reaction
PDH	Pyruvate dehydrogenase

PEP	Phosphoenolpyruvate
PEPCk	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PGPR	Plant Growth Promoting Rhizobacteria
POX	Pyruvate oxidase
PPC	Phosphoenolpyruvate carboxylase
PPP	Pentose phosphate pathway
PQQ	Pyrolloquinoline quinine
PSM	Phosphate solubilizing microorganisms
PTS	Phosphotransferase system
PYC	Pyruvate carboxylase
PYK	Pyruvate kinase
rpm	Revolutions per minute
Str	Streptomycin
Tc	Tetracycline
TCA	Tricarboxylic acid
Tmp	Trimethoprim
Tris	Tris (hydroxymethyl) aminomethane buffer
TYE	Tryptone Yeast Extract
WT	Wild type
YEMB	Yeast Extract Mannitol Broth
µl/ml	Microlitres/milliliters

Chapter 1

Review of Literature and Introduction

Introduction:

The world's plants, animals, fungi, and microbes are the working parts of Earth's life-support systems. Losing them imposes direct economic losses, lessens the effectiveness of nature to serve needs of human beings and carries significant economic losses. At least 40% of the world's economy and 80% of the needs of the poor are derived from biological resources (Dow and Downing, 2007). Soil is a biologically, physically, and chemically diverse entity that forms the basic substrate of terrestrial ecosystems, supports many human activities and provides a multitude of highly valuable ecosystem services (Dominati et al., 2010). The 'interface between the atmosphere and lithosphere, the outermost shell of the Earth' is defined as Soil by Bardgett (2005) which is formed over time. Soil microorganisms drive the biogeochemical processes that are the basis for life by interacting with plant roots and soil constituents at the root-soil interface, where root exudates and decaying plant material provide sources of carbon compounds for the heterotrophic biota. The number of bacteria in the rhizosphere (the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms) and rhizoplane (the external surface of roots together with closely adhering soil particles and debris) is higher than in the soil devoid of plants (Russo et al., 2012). Diverse group of soil microorganisms live in harmony amongst each other and with plants resulting into enhanced plant growth. Soil and its biota are essential for agricultural production, providing human beings with approximately 94% and 99% of protein and calories, respectively (FAOSTAT, 2003).

Plants are predominantly made up of carbon, oxygen and hydrogen which are supplied by air and water. Beyond these three elements, nitrogen is required in greatest quantity. The source of soil nitrogen is the atmosphere where nitrogen gas occupies about 79% of the total atmospheric gases. Living organisms that can fix nitrogen either symbiotically or free living are present in the soil and show profound effect on N content of soil. *Rhizobium* species are well known soil microorganisms which fixes nitrogen symbiotically with legumes by forming nodules (Shridhar, 2012).

1.1 *Rhizobium* : The Wonder Microbe

By the end of the 19th century, it was realized that atmospheric nitrogen was being assimilated through the root-nodules of legume plants. In 1888, Beijerinck reported isolation of the root nodule bacteria and established that they were responsible for this process of nitrogen fixation. He named these bacteria *Bacillus radicicola*. Later, Frank changed the name to *Rhizobium* with originally just one species, *R. leguminosarum*. They were the first biofertilizers produced and allowed savings of millions of dollars in chemical fertilizers which contaminate soil and water (Santos et al ., 2006).

1.1.1 General Taxonomy

In Bergey's original manual, bacteria showed nodulation capacity were termed as *rhizobia*, while bacteria with the same morphological characters that did not nodulate were excluded. Later rhizobial classification was based on growth and behavior with different host, further it was classified as either fast growing or slow growing (Vela´zquez et al., 2010). Recently, increased use of rhizobial species as microbial inoculants for legumes productivity has received a renewed attention for the identification from various hosts and locations. The current taxonomy of rhizobia consists of several genera in the subclass Alpha- and Beta- Proteobacteria. *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Azorhizobium*, *Methylobacterium*, *Bradyrhizobium*, *Phyllobacterium*, *Devosia* and *Ochrobactrum* are genera that belong to rhizobial Alpha-Proteobacteria. Rhizobial Beta-Proteobacteria includes the following genera: *Burkholderia*, *Herbaspirillum* and *Cupriavidus* (Dudeja and Narula, 2012).

1.1.2: Host Specificity and Nodulation

Rhizobium strain establishes a symbiosis with only a limited set of host plants, thus restricted number of bacteria nodulates leguminous plants (Mabrouk and Belhadj, 2010). Plants mutually compatible with the same species of rhizobia were listed in earlier years as cross inoculation groups (**Table 1.1**).

Table 1.1: Cross-inoculation group and *Rhizobium*-legume association (Morel et al., 2012).

Rhizobia	**Legume Cross-inoculation group**
Ensifer meliloti	Alfalfa Group: alfalfa (*Medicago sativa*), sweet clover (*Melilotus* spp.) (yellow and white), fenugreek (*Trigonella* spp.)
R. leguminosarum bv *trifolii*	Clover Group (Clover I, II, III and IV): clovers (*Trifolium* spp.)
B. japonicum	Soybean Group: soybean (*Glycine max*)
Bradyrhizobium spp.	Cowpea Group: pigeon pea (*Cajanus cajan*); peanut (*Arachis hypogaea*); cowpea, mungbean, black gram, rice bean (*Vigna* spp.); lima bean (*Phaseolus lunatus*); *Acacia mearnsii; A. mangium; Albizia* spp.; *Enterlobium* spp., *Desmodium* spp., *Stylosanthes* spp., Kacang bogor (*Voandzeia subterranea*), *Centrosema* sp., winged bean (*Psophocarpus tetragonolobus*), hyacinth bean (*Lablab purpureus*), siratro (*Macroptilium atropurpureum*), guar bean (*Cyamopsis tetragonoloba*), calopo (*Calopogonium mucunoides*), puero (*Pueraria phaseoloides*)
R. leguminosarum bv *viciae*	Pea Group: peas (*Pisum* spp.), lentil (*Lens culinaris*), vetches (*Vicia* spp.), faba bean (*Vicia faba*)
R. leguminosarum bv *phaseoli*	Bean Group: beans (*Phaseolus vulgaris*), scarita runner bean (*Phaseolus coccineus*)
Mesorhizobium loti	Chickpea Group: chickpea (*Cicer* spp.), Birdsfoot trefoil (*Lotus corniculatus* L.)
Rhizobium lupini	Group Lupines
Rhizobium spp.	Crown vetch

Establishment of effective symbiosis, between rhizobium and its host partner requires considerable amount of molecular communication for the development of nodules. Nod-factors or lipochito-oligosaccharide signaling molecules are central to the

initial establishment of legume- rhizobial symbiosis (Oldroyd and Downie, 2008; Madsen et al., 2011). Nod-factors are end products of the expression of the rhizobial nod genes. Many rhizobia produce more than one Nod-factor type molecule; probably a combination of Nod-factors is required for host recognition. Production of Nod-factors is activated by release of plant phenolic signals, predominantly flavonoids which activates set of nod genes in the compatible *rhizobial* strain (**Fig. 1.1** and **1.2**).

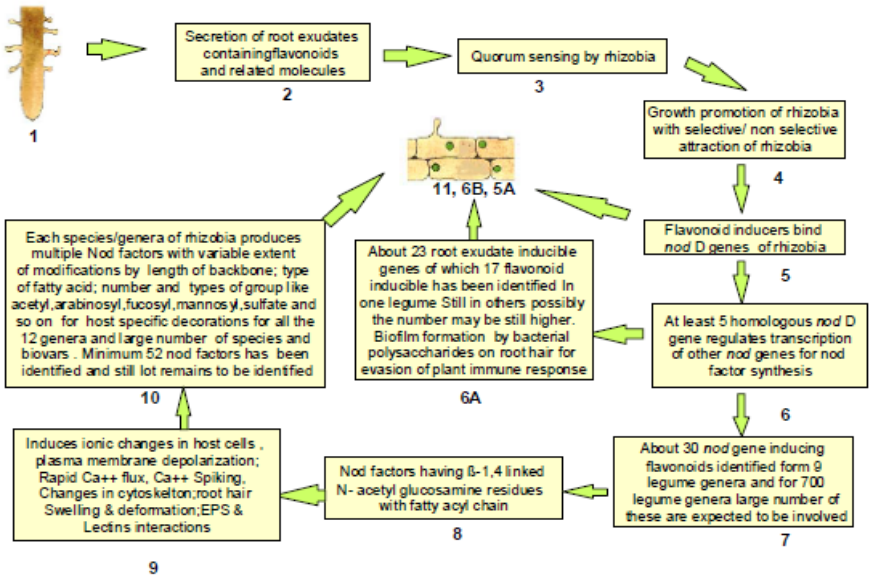

Fig. 1.1 Flavonoid, Nod factors and signal molecules exchange during nodule development.

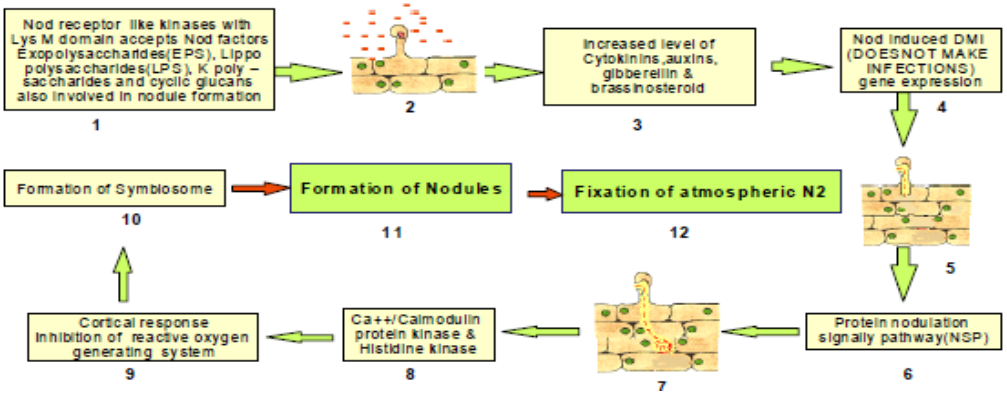

Fig. 1.2: EPS, phytohormones, endo-duplication, differentiation and development of functional nodules.

Nod factors are key molecules for legume symbiotic signaling and nodule organogenesis, additionally other rhizobial systems such as exopolysaccharide excretion, ethylene biosynthesis regulation, protein secretion systems and BacA are often required for the establishment of symbiosis with legumes as they help bacterial release into the host cytoplasm and bacteroid development (LeVier et al., 2000; Okazaki et al., 2004; Skorupska et al., 2006; Gresshoff et al., 2009; Deakin and Broughton 2009; Kouchi et al. 2010).

Legumes mainly develop two types of root nodules. Indeterminate nodules of legumes such as *Medicago truncatula* and *Pisum sativum* have a persistent meristem and are continuously infected. These nodules can be divided into four major zones (Popp and Ott, 2011). By contrast, determinate nodules (e.g. in legumes like *Lotus japonicus* and *Glycine max*) have a defined lifespan and lose their central meristem as well as the ability to be continuously infected upon maturation (summarised in **Fig. 1.3**).

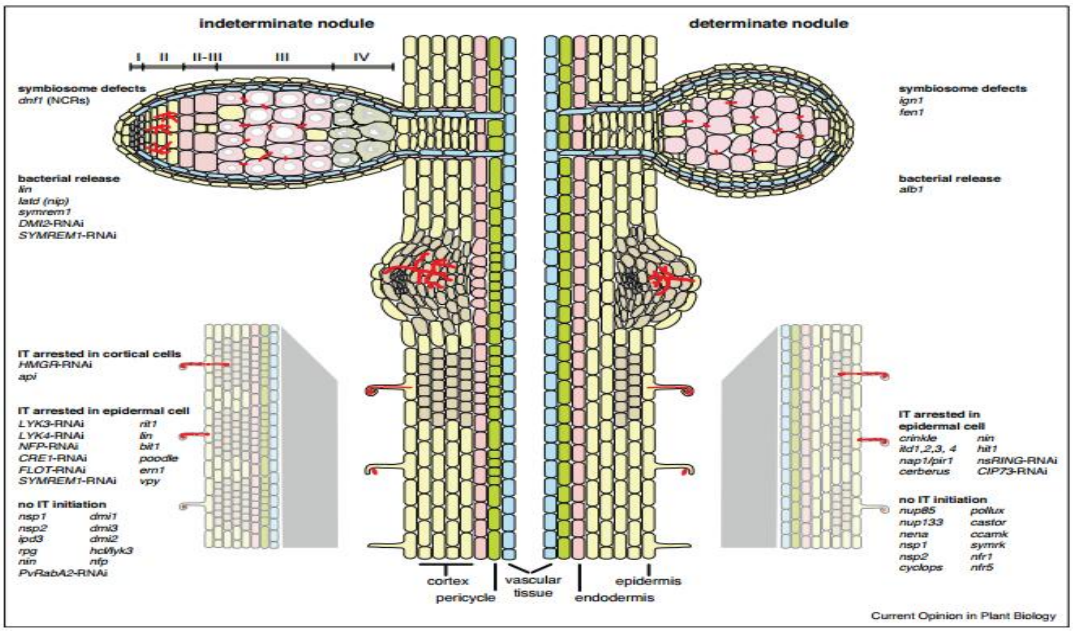

Fig. 1.3: Development of determinate and indeterminate root nodules (Popp and Ott, 2011).

Development of determinate and indeterminate root nodules and phenotypical stages associated with genes involved in rhizobial infection. In legumes that develop indeterminate nodules, Nod Factor perception induces periclinial cell divisions in the

pericycle followed by inner cortical cell proliferation during rhizobial infection. Development of a nodule primordium is accompanied by the presence of a persistent meristem leading to a zonation of an indeterminate nodule with the meristem (zone I), the infection zone (II), an interzone (II–III), the fixation zone (III) and the senescence zone (IV). By contrast, determinate nodules derive from cell divisions in the outer root cortex where meristematic activity is lost in mature nodules. A number of mutants in *M. truncatula* (indeterminate) and *L. japonicus* (determinate) have been identified that are impaired in perception of Nod Factors (NFs), rhizobial infection, bacterial release from infection threads (IT; red) or symbiosome formation (Popp and Ott, 2011). In the *Rhizobium*-legume symbiosis, the process of N_2 fixation strongly depends on the physiological state of the host plant. A competitive and persistent rhizobial strain will not be able to express its full N_2-fixation activity in presence of limiting factors or adverse environmental conditions (Mabrouk and Belhadj, 2010).

1.1.3: *Rhizobium* as nitrogen-fixer

Atmospheric N_2 is the main natural source of nitrogen, which makes up about 10% of the dry mass of biological matter. Biological Nitrogen fixation (BNF), is done by *Rhizobium* species, by enzyme Nitrogenase which converts atmospheric nitrogen into ammonia and thus breaks the strongest chemical bond in nature. The reaction it catalyzes is **(Fig.1.4).**

$$N_2 + 8\ e^- + 8\ H^+ + 16\ ATP \rightarrow 16\ ADP + 16\ P_i + 2\ NH_3 + H_2$$

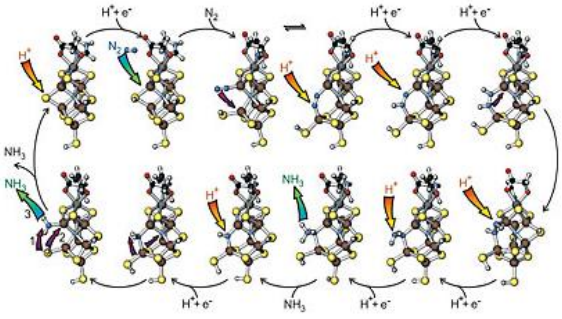

Fig. 1.4: Nitrogenase enzyme mechanism.

About 3.86 x 10^{18} kg nitrogen exists in the Earth's atmosphere. Every year N returned to the earth microbiologically is 1.39 x 10^{11} kg , where 65% (8.9 x 10^{10} kg) is contributed by legumes nodulated with *Rhizobium* species, remaining is by autotrophs or heterotrophs 'free' fixers (Shridhar, 2012). Along with N fixation *rhizobium* additionally enriches soil fertility through phosphate solubilization, improvement in nutrients and water uptake, synthesis of hormones, siderophores, to enhance plant growth and alleviate agro environmental problems (Badawi et al., 2011; Mader et al., 2011). The different N fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems are shown in **Fig. 1.5**.

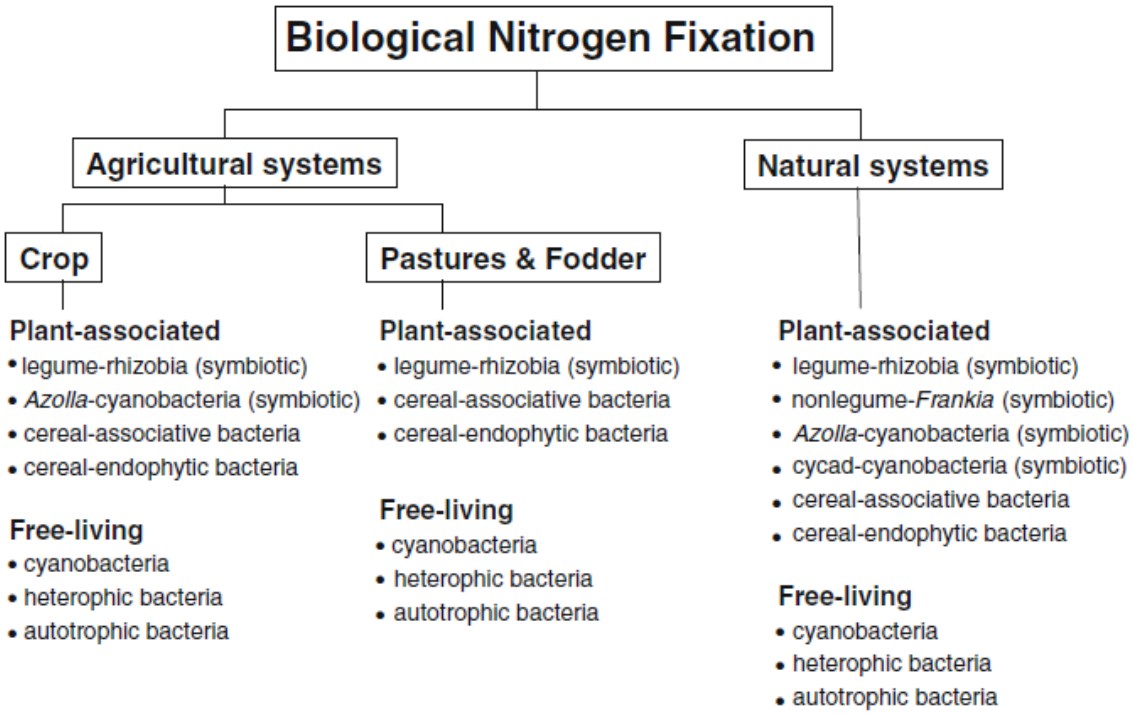

Fig. 1.5: The different N_2-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems (Herridge et al., 2008).

The use of legumes in crop rotations has been in use for centuries and is a major source of N in areas where the cost of fertilizer is too high or import is difficult (Bohlool et al., 1992; Crews and Peoples, 2004). Legumes allow other plants to benefit from this N obtained from the atmosphere. During the normal turnover and decomposition of legume roots, surrounding vegetation can access fixed N faster than through the breakdown of

above-ground plant tissues (Hardarson and Atkins, 2003). Based on this nitrogen-fixing symbiosis, legume crops require 35–60% less fossil-based energy than conventional, N-fertilized crops (Jensen et al., 2012). Thus, legumes are agriculturally and ecologically very important and account for 25% of the world's primary crop production (Ferguson et al., 2010).

1.1.4: Plant Growth Promoting Rhizobzcteria (PGPR)

The microorganisms of the rhizosphere include both deleterious and beneficial components that have the potential to influence plant growth and crop yield significantly. The beneficial rhizobacteria include the symbiotic rhizobia, certain actinomycetes and mycorrhizal fungi and free-living bacteria, increase the availability of nutrients or plant growth substances to plants and/or suppress parasitic and non-parasitic pathogens (Persello-Cartieaux et al., 2003). Plant growth promoting rhizobacteria (PGPR) are a group of free-living bacteria that colonize the rhizosphere and contribute to increased growth and yield of crop plants (Kloepper and Schroth, 1978). PGPR can affect plant growth by different direct and indirect mechanisms (Glick 1995; Gupta et al., 2000). Some examples of these mechanisms, which can probably be active simultaneously or sequentially at different stages of plant growth, are, increased mineral nutrient solubilization and nitrogen fixation, making nutrients available for the plant; repression of soil borne pathogens (by the production of hydrogen cyanide, siderophores, antibiotics, and/or competition for nutrients); improving plant stress tolerance to drought, salinity, and metal toxicity; and production of phytohormones such as indole-3-acetic acid (IAA) (Gupta et al. 2000). Moreover, some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of ethylene in plants (Glick et al., 1995). By lowering ethylene concentration in seedlings and thus its inhibitory effect, these PGPR stimulate seedlings root length (Glick et al. 1999) **(Fig. 1.6).**

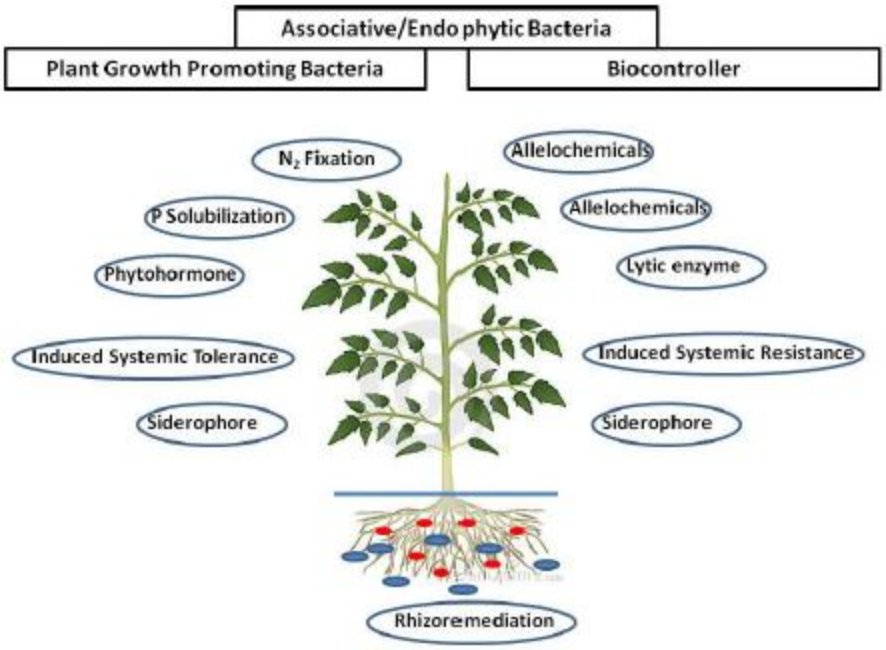

Fig.1.6: Properties of associative/endophytic bacteria for plant growth improvement (Jha et al., 2012).

1.1.4.1: *Rhizobium* as PGPR for non-legumes.

Rhizobia also have an excellent potential to be used as PGPR and PSM with non-legume plants (Chabot, 1996). Beyond nitrogen fixation, rhizobia also colonize roots of non-legume species and promote their growth without forming any nodule-like structure (Mehboob et al., 2009; Mia and Shamsuddin, 2010). Inoculation with *Rhizobium* had significant effect on the plant height, number of branches, root and shoot dry weight, number of nodule, seed and biomass yields, number of pod, crude protein rate and phosphorus content of seed (Erman et al., 2009). Increased rice production was seen by inoculation with a *Rhizobium leguminosarum* bv. trifolii strain (Yanni and Dazzo, 2010). *R. leguminosarum* PETP01 and TPV08 are excellent biofertilizers for tomato and pepper in different production steps leading to increased yield and quality (Garcı´a-Fraile1 et al., 2012). Rhizobia strains establish endophytic relationships with rice plants to promote shoot growth and enhance grain production (Biswas et al., 2000a, b; Mia and

Shamsuddin, 2010; Costa, et al., 2013). One native rhizobia, POA3 isolated from the Porto Alegre locality promoted growth of white clover (*Trifolium repens*) and rice plants (*Oryza sativa*) (Granada, et al., 2013).

The symbiotic rhizobia isolated from leguminous plants also promote plant growth *via* their inherent PGP capacities: siderophores and indolic compound production and nutrient solubilization (Ashraf et al., 2013; de Souza et al., 2013; Jida and Assefa, 2013). Indolic compounds production was the most common characteristic of the rhizobia species isolated from *Cajanus cajan* (Dubey et al., 2010). Crop enhancement, plant nutrients like P, K, Ca, Mg and even Fe accumulation and biofertilizer attributes were observed in cereal crops due to rhizobial inoculation (Mia and Shamsuddin, 2010). Rhizobia also promote plant growth by synthesis of vitamins, phytohormones and enzymes, producing siderophores, dissolving phosphates and other nutrients and prevention deleterious effects of phytopathogenic microorganisms besides biological nitrogen fixation (Boiero et al., 2007; Hayat et al., 2010; Ahemad and Khan, 2011).

The effects of *Rhizobium* inoculation, lime and molybdenum supply on yield and yield components of *Phaseolus vulgaris* L. significantly improved the number of pods per plant, number of seeds per plant, 100-seed weight and seed yield (Bambara and Ndakidemi, 2010). On average, an increase of 4-5% in crop yield has an important impact in agricultural production.

1.1.5: Effect of P on nodulation

Next to nitrogen, phosphorus is the most important element for adequate grain production. The evolution of science, particularly in the past century, has clearly demonstrated the significance of phosphorus for all animal and plant life on the earth (Ryan et al., 2012). Calcium increased root growth, number of nodule primordia, nodules, and growth of the soybean plant (Waluyo et al., 2013). Ca and P had a synergistic effect on BNF of soybean in acid soils. Ca is important for the establishment of nodules, whilst P is essential for the development and function of the formed nodules. P increased

number of nodule primordia, thus it also has an important role in the initiation of nodule formation. This effect of P supply on nodule formation is because P supply affects the production of root-exudates including flavonoids that trigger nod-gene expression to form nodules, and also plays a role in nodule cell metabolism that affects nodule development (Raghothama et al., 1999; Abel et al., 2002). Improved soil P status enhanced the positive effect of elevated [CO_2] on grain yield, biomass and shoot total N and P contents of the legumes tested (Lam et al., 2012). Besides contributing to plant growth by making soluble phosphorus more available, the legume-nodulating strains increased levels of soluble phosphate, thus improving the efficiency of biological nitrogen fixation (Silva et al., 2006). A positive correlation is observed between BNF and P availability in natural soils (Pearson and Vitousek, 2002; Labidi et al., 2003). Wherever soil P availability is low, elevated CO_2 does not increase BNF, and pasture quality decreased because of a reduction in above ground; at low P availability, there is a limited response of biomass production by grass community (Edwards et al., 2005; 2006).

Crop growth and yield reduce greatly due to low P availability especially for legumes, since legume nodules responsible for N_2 fixation have high P requirements (Sulieman and Tran, 2012). P is often the limiting element for biological productivity (Sato and Miura, 2011; Lopez-Arredondo and Herrera-Estrella, 2012). 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*). Plants engaged in symbiotic N_2 fixation have high P demand (Vance et al., 2003; Sulieman et al., 2013a). Low levels of phosphorus affected symbiosis by decreasing the supply of photosynthates to the nodule, which reduced the rate of bacterial growth and the total population of legume-nodulating microorganisms (Moreira et al., 2010). P availability dominantly controlled free-living N fixation in tropical rain forest (Reed et al., 2013). Thus, the efficiency of nitrogen fixation by the strains approved as inoculants may be related to a greater ability to solubilize low soluble phosphates.

1.1.6: Phosphorus deficiency and Nitrogen fixation

Phosphorus deficiency is commonly reported along with Al^{3+} toxicity as 40% of the world's arable soil is considered acidic. Phosphorus deficiency and Aluminium (Al^{3+}) toxicity are associated with each other in acid soils, and they both have major effects on legume plant growth and function and are collectively considered as inseparable factors that limit crop productivity on such soils (Ward et al., 2008). Nodule biomass is strongly correlated to P availability to plants as about 3 times more P is required by nodules than the surrounding root tissues. An increase in P supplied to host legume plants led to a 4-fold increase in nodule mass (Olivera et al., 2004). P deficiency in soil severely limits plant growth productivity, in legumes, and this has a deleterious effect on nodule formation, development and function (Haque et al., 2005). Nodule construction cost and growth respiration of soybeans increased with P deficiency (Andrews et al., 2009). In the case of legumes, more P is required by symbiotic than non-symbiotic plants. Symbiotic nitrogen fixation (SNF) has a high demand for P, with up to 20% of total plant P being allocated to nodules during N_2 fixation. The process consumes large amounts of energy, such that the energy generating metabolism is depended upon the availability of P (Schulze et al., 1999; Schenk, 2012). The effects of P deficiency may be direct, as P is needed by nodules for their growth and metabolism, or indirect. The high requirement of P are linked to its role in nodule carbon and energy metabolism, therefore as the deficiency may affect the supply of carbon to the nodules, the bacteria will have greater respiratory demand on the host plant during nitrogen fixation (Sar and Israel, 1991; Valentine et al., 2011).

1.1.7: *Rhizobium*- PSM co-inoculation

Considering the main limitations to the biological N_2 fixation with soybeans and common beans inoculated with rhizobia and the benefits to crop growth attributed to *Azospirillum*, co-inoculation with both microorganisms might improve plant's performance. This approach is current with modern demands of agricultural, economic, social and environmental sustainability (Chaparro et al., 2012). Many evidences are there

to show that co-inoculation with *Rhizobium* and PSM have additive or synergistic effect on plant growth and crop yield (**Table 1.2**) (Morel and Brana, 2012). The results confirm the feasibility of using rhizobia and PSM such as azospirilla as inoculants in a broad range of agricultural systems, replacing expensive and environmentally unfriendly N-fertilizers (Hungria et al., 2013).

Table 1.2: Ten years of studies on legume co-inoculation (2002-2012). Increase in legume symbiotic parameters and yield by co-inoculation compared to single-inoculation with rhizobia. Abbreviations are as follows: RDW: root dry weight; SDW: shoot dry weight; RL: root length; NN: nodule number; NFW: Nodule fresh weight; PDW: plant dry weight; PFW: plant fresh weight (Morel et al., 2012).

Rhizobium and PSM	**Host plant**	**Observation (% increase)**	**Reference**
Rhizobium and PSB	chickpea	enhanced nodulation, plant growth, yield and nutrient uptake	Rudresh et al., 2005.
R. leguminosarum D293 and AM fungi	Pea	increased plant biomass, nodulation parameters, N_2 fixation activity, increased significantly total P content in plant tissues and percentage of root colonization.	Stancheva et al., 2006.
Bradyrhizobium sp. (*Vigna*) and *B. subtilis*	green gram plants	increased dry matter yield, chlorophyll content in foliage and N and P uptake	Zaidi and Khan, 2006
Bradyrhizobium spp./ *Rhizobium* and PGPR	legumes	increased root and shoot biomass, nodule dry matter, nitrogenase activity, N_2-fixation, and grain yield.	Elkoca et al., 2008.
B. japonicum -P. putida	Mung bean (*Vigna radiata*	Increase in total Biomass and in Nodule Number	Shaharoona et al., 2006
Rhizobium sp. -*P. putida/ P. fluorescens/ B. cereus*	Pigeon pea (*Cajanus cajan*)	Increase in Nodule Number	Tilak et al., 2006
Rhizobium sp.-*Bacillus* spp.	Pigeon pea (*Cajanus cajan*)	Increase in plant fresh weight and in Nodule Number	Rajendran et al., 2008
R. leguminosarum-B. thuringeinsis	Lentin (*Lens Culinaris L.)*	Increase in plant fresh weight and in Nodule Number	Mishra et al., 2009

R. leguminosarum-B. thuringeinsis	Pea (*Pisum sativum* L. cv. Capella	Increase in plant fresh weight and in Nodule Number	Mishra et al., 2009
R. leguminosarum bv viceae -P. fluorescens	Pea (*Pisum sativum* L. cv. Capella	Increase in plant dry weight	Kumar et al., 2001
R. leguminosarum bv. *viciae - A. brasilense* 30	Vetch (*Vicia sativa*)	Increase in SDW, nod gene induction and decrease in indoles content	Star et al., 2011
R. galegae bv. *orientalis* - *Pseudomonas* spp.	Galega (*Galega orientalis*)	Increase in SDW RDW and in Nodule Number	Egamberdieva et al., 2010
S. meliloti - Delftia sp.	Alfalfa (*Medicago sativa*)	increase in SDW and in nodulation rate	Morel et al., 2011
Bradyrhizobium sp. - *Pseudomonas* sp./ *Ochrobactrum cytisi*	Altramuz (*Lupinus luteus*)	66 in SDW and 20-40, 25, and 30-50 decrease in Cd, Cu and Zn – accumulation in roots, respectively	Dary et al., 2010
R. leguminosarum bv.trifolii - P. fluorescens	Clover (*Trifolium repens*)	20 in SDW; 100 in Nodule Number	Marek-Kozaczuk and Skorupska, 2001
R. leguminosarum bv. trifolii - Delftia sp	Clover (*Trifolium repens*)	50 in SDW and 80 in nodulation rate	Morel et al., 2011
Rhizobium sp. -	Peanut (*Arachis*	50 in PDW; 80 in Nodule Number	Anandham et al., 2007

Thiobacillus sp.	*hypogaea*)		
M. ciceri - Azotobacter chroococcum	Chickpea (*Cicer arietinum*)	15 in Nodule Number; 25 in P-soil availability	Qureshi et al., 2009
M. ciceri - Pseudomonas sp/ *Bacillus* sp	Chickpea (*Cicer arietinum*)	20 in PDW; 30 in Nodule Number; 100 in P-uptake	Wani et al., 2007
Mesorhizobium sp. *Cicer - Pseudomonas* spp.	Chickpea (*Cicer arietinum*)	70 in Nodule Number; 30 in SDW, 30 in Nuptake	Goel et al., 2002
Mesorhizobium sp. *Cicer -Pseudomonas* spp.	Chickpea (*Cicer arietinum*)	1,2-1,86 in Nodule Number; 1,3-2,11 NFW; 1-2,93 in PDW	Malik and Sindhu, 2011
Rhizobium -B. subtilis/ megaterium	Chickpea (*Cicer arietinum*)	18 in SDW; 16-30 in RDW; 14 in total biomass yield	Elkoca et al., 2008
Rhizobium spp. -*A. brasilense*	Common bean (*Phaseolus vulgaris*)	30 total yield	Remans et al., 2008b
R. tropici - Paenibacillus Polymyxa	Common bean (*Phaseolus vulgaris*)	50 in Nodule Number; 40 in N uptake in non-drought stress	Figuereido et al., 2008
R. tropici/etli - A.	Common bean	18-35 and 20-70 in RDW; 29 and 28 in SDW under non	Dardanelli et al., 2008

brasilense	(*Phaseolus vulgaris*)	saline and saline conditions, respectively.	
R. etli - C. balustinum	Common bean (*Phaseolus vulgaris*)	35 in SDW; 35 in Nodule Number under non-saline conditions; and 39 in SDW; 63 in RDW under saline conditions	Estevez et al., 2009
Rhizobium spp. - *P. putida /B. subtilis/A. brasilense*	Common bean (*Phaseolus vulgaris*)	30 in Nodule Number; 20 in SDW; 30-45 in RDW	Remans et al., 2007
Rhizobium spp. - *A. brasilense*	Common bean (*P. vulgaris*)	70 in Nodule Number	Remans et al., 2008a
R. tropici - *Paenibacillus polymyxa*	Common bean (*Phaseolus vulgaris*)	50 in Nodule Number; 40 in N uptake in non-drought stress	Figuereido et al., 2008
Rhizobium spp. - *P. fluorescens /A. lipoferum*	Common bean (*Phaseolus vulgaris*)	25 in Nodule Number; 13 in SDW; 74 in seed yield	Yadegari et al., 2010
S. meliloti B399 and the *Bacillus* sp. M7c	alfalfa plants	increase in root /shoot dry weight, length, surface area of roots, number, and symbiotic properties	Guiñazú et al., 2010; Lorena et al 2010.
E. fredii - *Chryseobacterium balustinum*	Soybean (*Glycine max*)	56 and 44 in SDW; 100 and 200 in RDW; 155 and 286 in Nodule Number	Estevez et al., 2009

B. japonicum - *P. putida*	Soybean (*Glycine max*)	40 in SDW; 80 in Nodule Number; 45 in RDW	Rosas et al., 2006
B. japonicum - *B. subtilis/ S. proteamaculans*	Soybean (*Glycine max*)	12 in SDW; 10 in P-uptake	Han and Lee, 2005
B. japonicum - *A. brasilense*	Soybean (*Glycine max*)	47 in Nodule Number	Cassán et al., 2009
Rhizobium and PSB	grass	enhanced nodulation and increased the number and weight of nodules	Abusuwar and Omer, 2011.
Pseudomonas fluorescens P-93 /*Azospirillum Lipoferum S-21*, *Rhizobium* strains Rb-133 and Rb-136	Common bean seeds	Increased nodule number and dry weight, shoot dry weight, amount of nitrogen fixed as well as seed yield and protein content.	Yadegari et al., 2010
B. japonicum RCR 3407 strain *B. subtilis*	Common bean seeds	influence plant growth, vitality, and the ability of the plant to cope with pathogens	Elkoca et al., 2010; Tsigie et al., 2012
Rhizobium /PSB	faba bean plants	increased yield and seed quality decreased seeds carbohydrate content	Rugheim and Abdelgani, 2012
Pseudomonas and *Rhizobium* isolates	common bean	improved growth and yield production	Samavat et al., 2012

Rhizobium and AM fungi	Chick pea plant	Significantly increased fresh and dry weights of shoot and root..	Moradi et al., 2013
P. chlororaphis and A. pascens amendment with RP	Walnut	Highest plant height, shoot /root dry weight, P / N uptake of walnut seedlings, the maximum amounts of available P and N in soils.	Xuan Yu et al., 2012
B. japonicum with *A. brasilense*	Soybean and common bean	increased seed yield, improved nodulation	Hungria et al., 2013
tetra inoculants *R. leguminosarum* + *A. chroococcum* + *P. aeruginosa* + *T. Harzianum*, tri inoculants of *R. leguminosarum* + *A. chroococcum* + *P. aeruginosa* and *R. leguminosarum* + *A. chroococcum* + *T. harzianum*	Common bean seeds	Significant nodulation, grain yield, and nutrient uptake.	Varma and Yadav, 2012

1.1.8: Phosphate solubilization by *Rhizobium* spp.

The phosphate-solubilizing activity of *Rhizobium* is associated with the production of 2-ketogluconic acid, indicating that phosphate-solubilizing activity of the organism is entirely due to its ability to reduce pH of the medium (Halder and Chakrabarty, 1993). Since 1950s it is reported that P-solubilizing bacteria release phosphorus from organic and inorganic soil phosphorus pools through mineralization and solubilization **(Fig. 1.7)** (Khan et al., 2009).

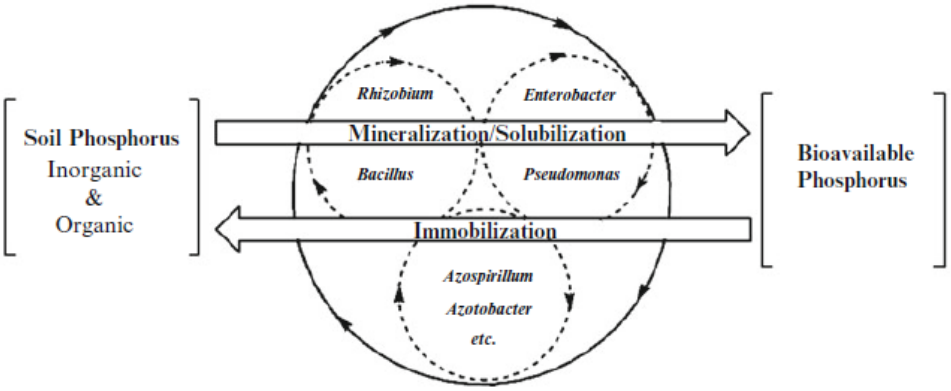

Fig.1.7: Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan et al., 2009)

P solubilization and mobilization in soils has been shown in **Fig. 1.8.** A wide range of microbial P solubilization mechanisms exist in nature and much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi (Banik and Dey, 1982). Phosphatic rocks are solubilized by acid producing microorganisms to release more P for plant uptake (Gyaneshwar et al., 2002). A few strains or species of *Rhizobium* are involved in phosphate solubilization also along with symbiotic nitrogen fixation (Deshwal et al., 2003). Lowering of soil pH by microbial production of organic acids such as acid phosphatases, lactate, citrate, and succinate, gluconic and 2-ketogluconic acids etc. and proton extrusion is the main principal mechanism of mineralization of organic form of phosphorus (Goldstein, 1995; Deubel et al., 2000). Phosphate availability in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes (Alikhani et al., 2006). Many phosphate-solubilizing bacteria (PSB) are found in soil and in plant rhizospheres and potentially represent 40% of the culturable population (De Freitas, et al., 1997; Richardson, 2000; Chen et al., 2006). PSB produce a range of organic acids such as

citrate, lactate, and succinate that solubilize mineral phosphates. To make P available for plant nutrition *Bacillus, Pseudomonas, Klebsiella* and *Enterobacter* spp. are involved in the stepwise degradation of phytate to lower phosphate esters of myo-inositol and phosphorous by means of acid and alkaline phosphatase enzymes (Podile and Kishore, 2006). Bacteria also enhance phosphorus availability to crops by solubilizing precipitated forms of phosphorus (Chen et al., 2006). Single and dual inoculation with *Rhizobium* along with P fertilizer is 30-40% better than only P fertilizer for improving grain yield of wheat, where *Rhizobium* with non-legumes could act as phosphate solubilizer, hormone producer and to some extent as N-fixer (Afzal and Bano, 2008). Bacteria assimilate soluble phosphorus, and make it available by preventing it from adsorption (Khan and Joergensen, 2009). Phosphate solubilization activity of rhizobia is related with the production of 2-ketogluconic acid, due to its ability to reduce pH of the medium (Hayat et al., 2010). During phosphate solubilization, the nature of organic acid produced by rhizobia is more important than the quantity.

Rhizobium ciceri inoculation and phosphorus application in combination increased growth rate and P utilization of chickpea cultivars as compared to the control, greatly affected the P Efficiency Index (EI) and P utilization performance of chickpea cultivars.(Karaman et al., 2013). Certain strains of *R. leguminosarum* (bv. viciae, bv. phaseoli, bv. trifolii), *R. leguminosarum* sp, *B. japonicum, Mesorhizobium ciceri, Mesorhizobium mediterraneum* and *S. meliloti* are good P-solubilizers (Antoun et al., 1998; Peix et al., 2001; Alikhani et al., 2006; Daimon et al., 2006; Rivas, 2006; Boiero et al., 2007). *B. japonicum* 518 strain showed the ability to solubilize insoluble tricalcium phosphate (Marinkovic et al., 2013).

1.1.9: Phosphorus in agriculture

Phosphorous is going to be plant nutrient that will limit the agricultural production in the next millennium**.** It is a major growth-limiting nutrient, and unlike the case of nitrogen, there is no large atmospheric source that can be made biologically available (Ezawa et al., 2002). As regards the role of P, it stimulates root development and growth, gives plant rapid and vigorous start leading to better tillering, essential for many metabolic processes in plant life and for seed formation and organization of cells, encourages earlier maturity. In most soils, its content is about 0.05% of which only 0.1% is plant available

(Achal et al., 2007). About 20-25% of total phosphorous in arid soils of India is organic in nature and 68% organic phosphorous in the soil is present as phytin (Yadav and Tarafdar, 2007), which are not directly available to plants. Phosphorous is taken up from soil in the form of soluble orthophosphate ions; $H_2PO_4^{-1}$, HPO_4^{-2} and PO_4^{-3} and generally the availability of these ions to the plants is in the order of $H_2PO_4^{-1}$>HPO_4^{-2} > PO_4^{-3}. Only about 20% of the phosphorus used in agriculture reaches the food we consumed, most of the rest is lost in inefficient steps along the phosphorus cycle (Cordell et al, 2011) (**Fig. 1.8**).

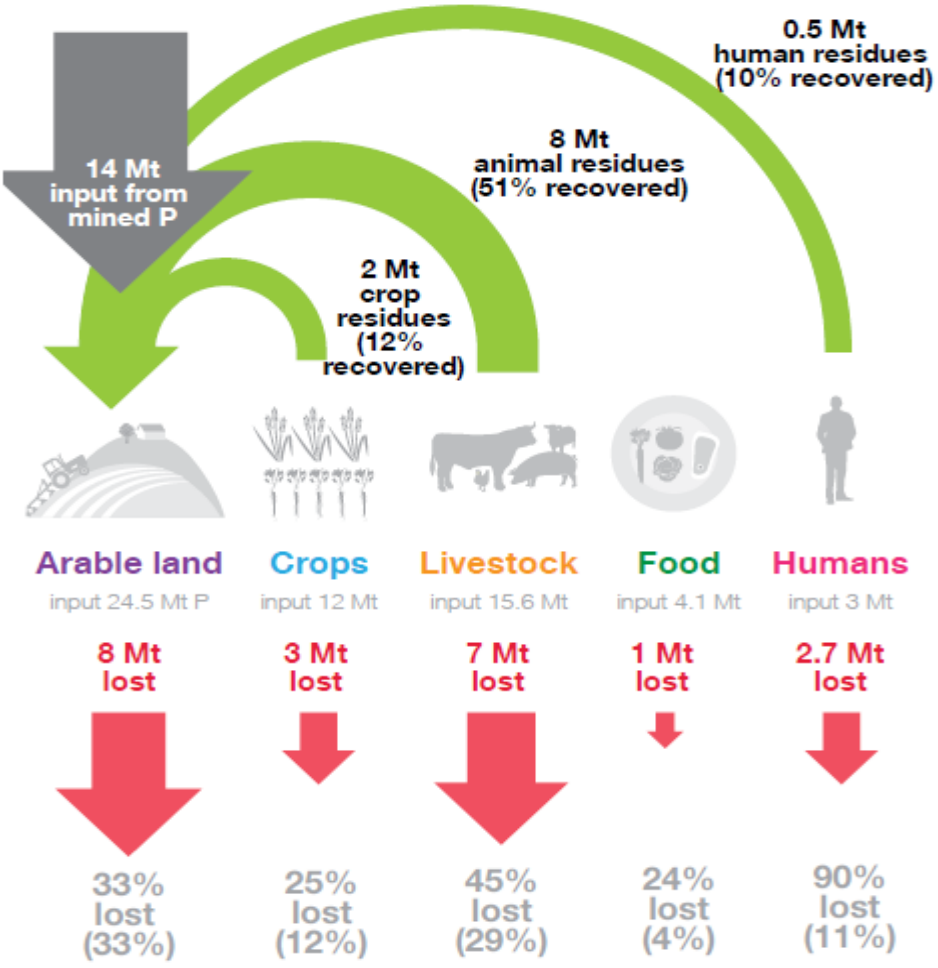

Fig. 1.8: Simplified cycle of phosphorus in agriculture (based on data from Cordell et al., 2009 and 2011). Red arrows represent losses into water systems ultimately, and green arrows represent current recoveries into arable land from the different subsystems. The percentages under the red arrows represent the percentage losses from each subsystem, and shown in brackets are the percentage losses relative to the total input into agriculture land. For example, the livestock system loses about 45% of the phosphorus entering the livestock

system itself, and this represents about a 29% loss of the phosphorus entering the agriculture system overall.

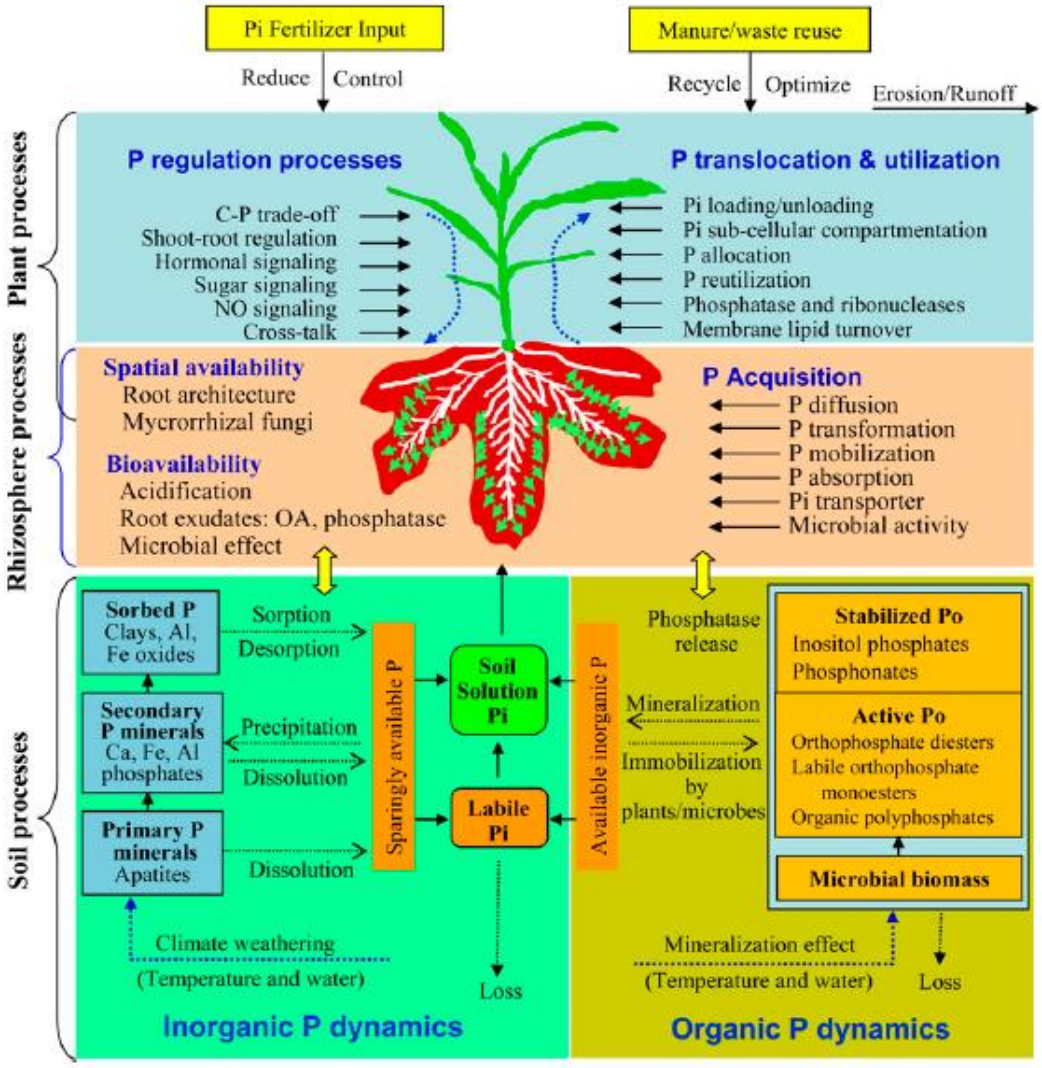

Fig. 1.9: P dynamics in the soil/rhizosphere-plant continuum C-P, Carbon- P; NO, nitric oxide; OA, organic acids (Shen et al., 2011).

Rhizobacteria secrete organic acids as end products or by-products of primary metabolism. In most cases, sugars are catabolized by glycolytic or Entner-Doudroff pathway. The amount of the organic acid secretion differs between members of the same genus and sometimes between strains of the same species due to presence or absence of enzymes (Vyas and Gulati, 2009; Buch et al., 2010). Organic acids of aerobic or anaerobic

respiration such as gluconic acid, 2-ketogluconic acid are directly formed extracellularly or in the in the periplasm by the membrane bound enzymes (**Fig. 1.10)** (Archana et al., 2012). However, organic acids formed by intracellular enzymes require specific transport proteins that aid in their extracellular secretion. Mono-, di- and tri-carboxylate transporters are located in the plasma membrane mediate their secretion.

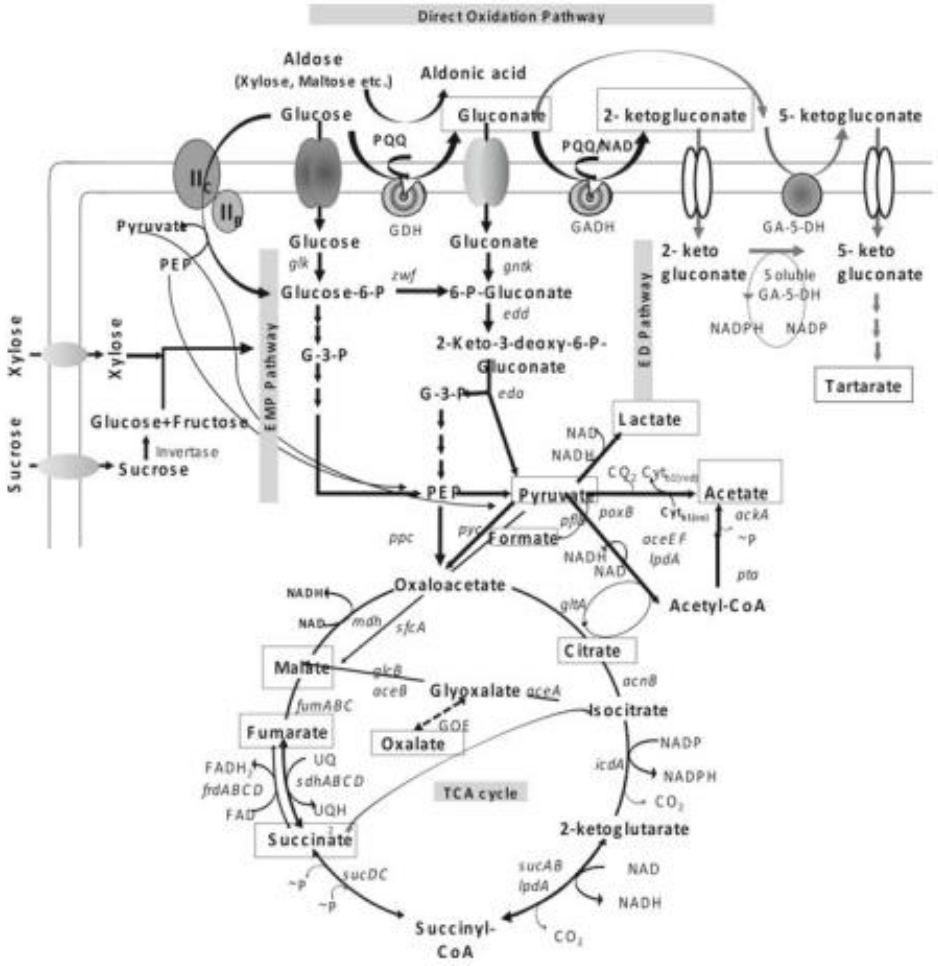

Fig. 1.10: Pathways and enzymes involved in organic acid biosynthesis by rhizobacteria (Archana et al., 2012). The organic acids secreted are depicted in boxes. The diagram depicts a comprehensive set of pathways – all may not be present in any given organism. Abbreviations: GDH glucose dehydrogeanse, GADH gluconate dehydrogenase, GA-5-DH gluconate-5-dehydrogenase, glk Glucokinase, zwf Glucose-6-phosphate dehydrogenase, gntk Gluconate kinase, edd 6-phosphogluconate dehydratase, eda 2-keto-3-deoxy-6-phosphogluconate aldolase, ppc phosphoenolpyruvate carboxylase, pyc pyruvate carboxylase, gltA citrate synthase, acnB Aconitase, icdA Isocitrate dehydrogenase, icl Isocitrate lyase, sucABa ketoglutarate dehydrogenase, sucDC succinyl-CoA synthetase, sdhABCD succinate dehydrogenase, fumABC Fumarase, frdABCD fumarate reductase,

mdh Malate dehydrogenase, sfcA malic enzyme, aceA Isocitrate lyase, aceB/glcB Malate synthase, GOE Glyoxalate oxidizing enzyme, ldh Lactate dehydrogenase, aceEF-lpdA pyruvate dehydrogenase, pta phosphotransacetylase, ackA acetate kinase A, poxB pyruvate oxidase, pfl pyruvate formate lyase.

Table 1.3: Organic acids involved in P-solubilization and produced by PS bacteria (Zaidi et al., 2009).

Bacterial communities	**Organic acids produced**	**References**
Burkholderia cepacia DA23	Gluconic	Song et al. (2008)
Pseudomonas corrugata (NRRL B-30409)	Gluconic, 2-ketogluconic	Trivedi and Sa (2008)
Citrobacter sp. DHRSS	Acetic, gluconic	Patel et al. (2008)
Burkholderia, Serratia, Ralstonia and Pantoea	Gluconic	Elizabeth et al. (2007)
Bacillus, Rhodococcus, Arthrobacter, Serratia and one Chryseobacterium, Delftia, Gordonia, Phyllobacterium, Arthrobacter ureafaciens, Phyllobacterium myrsinacearum, Rhodococcus erythropolis and Delftia sp.	Citric, gluconic, lactic, succinic, propionic	Chen et al. (2006)
Enterobacter intermedium	2-ketogluconic	Hwangbo et al. (2003)
B. amyloliquefaciens, B. licheniformis, B. atrophaeus, Penibacillus macerans, Vibrio proteolyticus, xanthobacter agilis, E. aerogenes, E. taylorae, E. asburiae, Kluyvera cryocrescens, P. aerogenes, Chryseomonas luteola	Lactic, itaconic, isovaleric, acetic isobutyric	Vazquez et al. (2000)
Pseudomonas cepacia	Gluconic, 2-ketgluconic	Bar-Yosef et al. (1999)
Bacillus polymyxa, B. licheniformis,	Oxalic, citric	Gupta et al. (1994)

Bacillus spp.		

Table 1.4 Organic acids produced by phosphate solubilizing fungi (Zaidi et al 2009)

Organism	**Organic acids produced**	**References**
Aspergillus niger	Gluconic, oxalic	Chuang et al. (2007)
Penicillium oxalicum	Malic, gluconic, oxalic	Shin et al. (2006)
Aspergillus flavus, A. niger, Penicillium canescens	Oxalic, citric, gluconic succinic	Maliha et al. (2004)
Penicillium rugulosum	Citric, gluconic	Reyes et al. (2001)
A. niger	Succinic	Vazquez et al. (2000)
Penicillium variabile	Gluconic	Fenice et al. (2000)
Penicillium rugulosum	Gluconic	Reyes et al. (1999)
Penicillium radicum	Gluconic	Whitelaw et al.(1999)
P. variabile	Gluconic	Vassilev et al. (1996)
A. niger	Citric, oxalic, gluconic	Illmer et al. (1995)
A. awamori, A. foetidus, A. tamari, A. terricola, A. amstelodemi,	Oxalic, citric	Gupta et al. (1994)
A. japonicus, A. foetidus	Oxalic, citric gluconic succinic, tartaric	Singal et al. (1994)

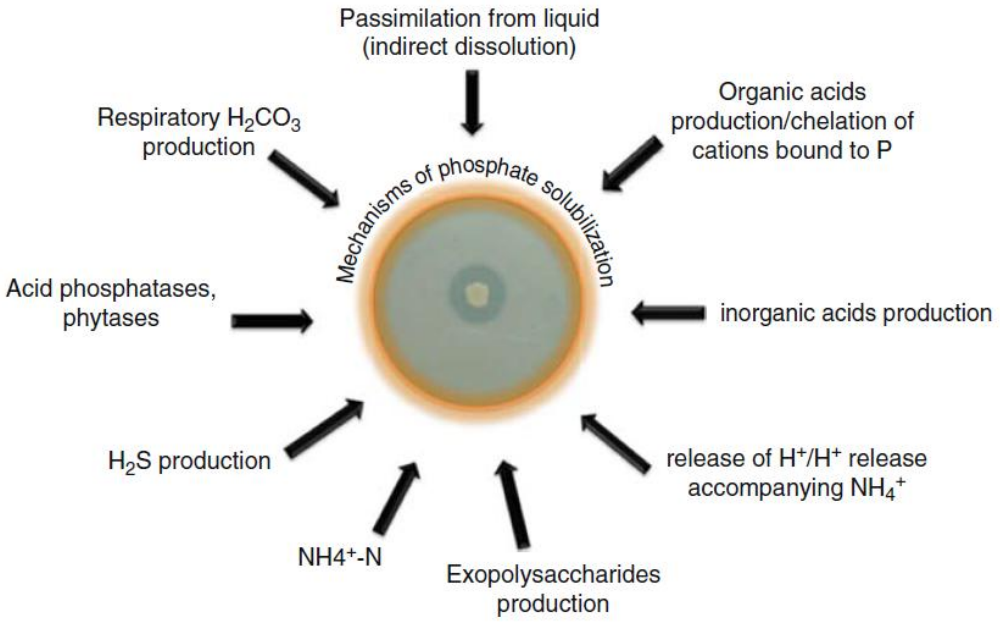

Fig. 1.11: Mechanisms of P-solubilization by phosphate solubilizing bacteria (Zaidi et al., 2009).

PS microbes are well known for making soluble P accessible for uptake by plants. They can also facilitate growth and development of plants by producing essential nutrients (Thomas et al., 2005) or by changing the concentration of plant growth promoting substances including phytohormones such as indoleacetic acid (Wani et al., 2007a, b), through asymbiotic or symbiotic N_2 fixation (Zaidi, 2003; Zaidi and Khan, 2007), soil conditioning, exhibiting bio-control activity (Pandey et al., 2006), by synthesizing siderophores (Vassilev et al. 2006), antibiotics, and cyanide (Lipping et al., 2008), by synthesizing an ACC deaminase that can modulate plant ethylene levels (Anandham et al., 2008; Poonguzhali et al., 2008), and by solubilizing or reducing the toxicity of metals (bioremediation) (Khan et al., 2009).

1.2: Glucose metabolism in Various Organisms.

1.2.1: Glucose catabolic pathways in pseudomonads

In pseudomonads although organic acids are the preferred carbon sources presence of glucose as the sole carbon source does induce the glucose metabolizing pathways. Pseudomonads do not catabolize glucose to triose phosphate via the traditional EMP pathway as they lack the key glycolytic enzyme PFK (Lessie and Phibbs, 1984). Unlike *E.*

coli, pseudomonads generally lack PEP-PTS system for glucose uptake (Romano et al., 1970). Instead, pseudomonads catabolize glucose by two different routes: the direct oxidative pathway which acts on glucose extracellularly and the simultaneously operating intracellular phosphorylative pathway. Pseudomonads glucose oxidation occurs in two successive reactions forming D-gluconate and 2-keto-D-gluconate (2-KG) catalyzed by a membrane-bound PQQ-GDH and gluconate dehydrogenase (GADH) respectively (Lessie and Phibbs, 1984; Fuhrer et al., 2005) **(Fig.1.12).**

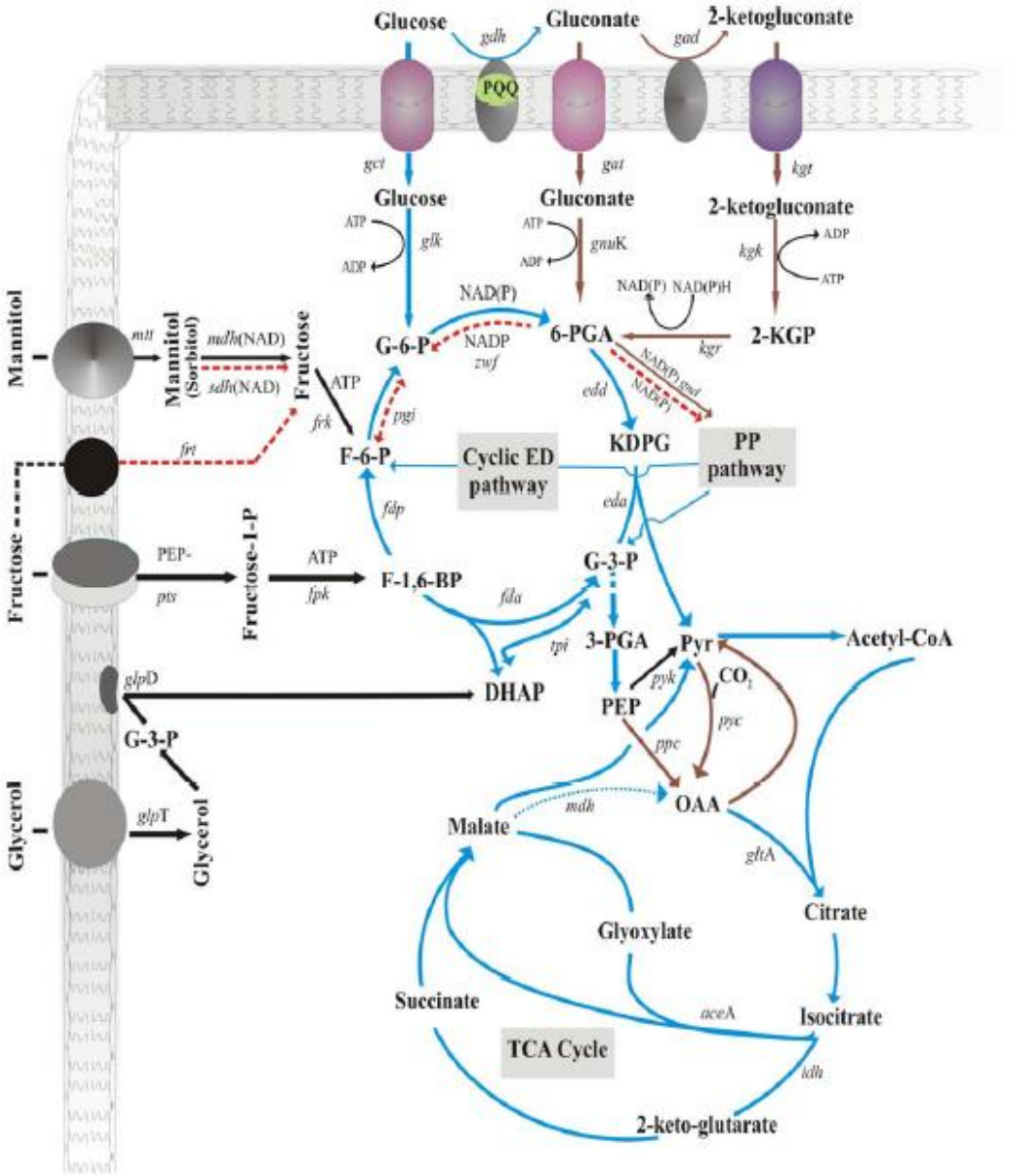

Fig. 1.12: Carbohydrate metabolism in pseudomonads. Key to the pathway: Blue lines/arrows=Pathway operating in presence of glucose; **Bold Black arrows**=Reactions occurring when carbon source is other than glucose; Blue dashed arrow=Flux through that

reaction is very low; Brown arrows=Occurrence of those genes is highly variable from strain to strain; Red dashed arrows=Reactions specific to *Pseudomonas cepacia*.

1.2.2: Glucose Metabolism in *Bacillus subtilis*

B. subtilis is a gram positive spore forming bacterian and is the second most intensively studied bacteria after *E. coli.* Glucose is internalized via PTS *and* metabolizes a large proportion of it to pyruvate and acetyl CoA, and subsequently converts these compounds to lactate, acetate and acetoin as by-products of metabolism which are excreted into the extracellular environment. The overall flux distribution done by ^{13}C metabolic flux analysis suggested glycolysis as the main catabolic pathway for glucose, acetate secretion, significant anaplerosis, and absent gluconeogenesis (**Fig. 1.13**) (Martin et al., 2011).

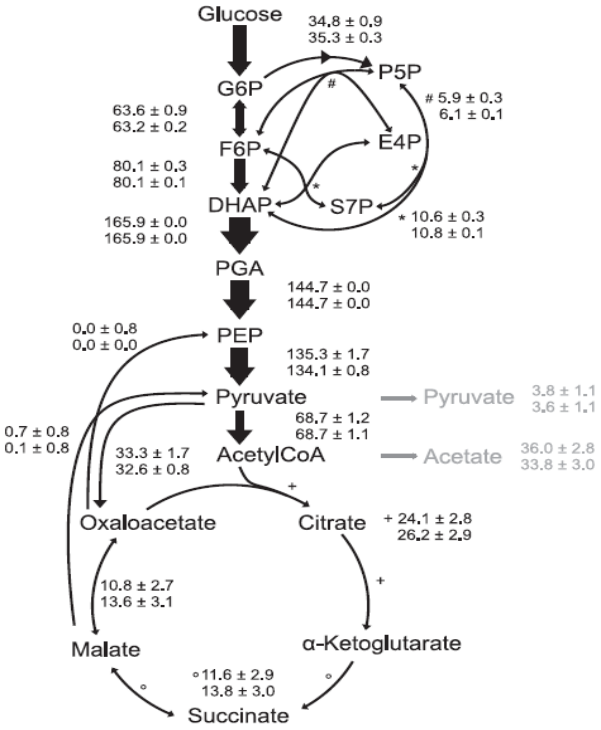

Fig. 1.13: Glucose Metabolism in *Bacillus subtilis* (Martin et al., 2011). Shown are relative flux values normalized to the glucose uptake rate of 8.2mmol g-1 h-1. Black arrows depict maximum and inner white arrows the minimum estimated flux value based on the Monte Carlo bootstrap error estimates with a confidence interval of 95%.

1.2.3: Glucose Metabolism in *Rhizobium* (Stowers et al., 1985).

The mechanism of glucose transport is established in both fast and slow-growing rhizobia, neither fast nor slow-growing rhizobia possessed a phosphoenolpyruvate

phosphotransferase system and the uptake of glucose proceeded via an active process requiring an energized membrane state (Stowers et al., 1977; Mulongoy et al., 1978; DeVries et al., 1982). Carbohydrate supply is a major factor limiting nitrogen fixation by the *Rhizobium*-legume symbiosis (Bethlenfalvay and Phillips, 1977; Hardy, 1977; Pate, 1977). Both fast- and slow-growing species possess the Entner-Doudoroff pathway (Katznelson and Zagallo, 1957; Keele et al., 1969; Martinez-de Drets and Arias, 1972; Mulongoy and Elkan, 1977 a). Fast-growing rhizobia also possess $NADP^+$-dependent 6-phosphogluconate dehydrogenase the key enzyme of the pentose phosphate pathway, but it was not found in slow-growing rhizobia (Katznelson and Zagallo, 1957; Keele et al., 1969; Martinez-de Drets and Arias, 1972; Mulongoy and Elkan, 1977a, b). The tricarboxylic acid cycle also operated in hexose catabolism in *B. japonicum* (Keele et al., 1969; Mulongoy and Elkan, 1977a). ED pathway was established as the presence of 6PG dehytratase (EC 4. 2. 1. 12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) activities were observed in glucose-grown cells (Keele et al.,1970; Stowers et al., 1985).

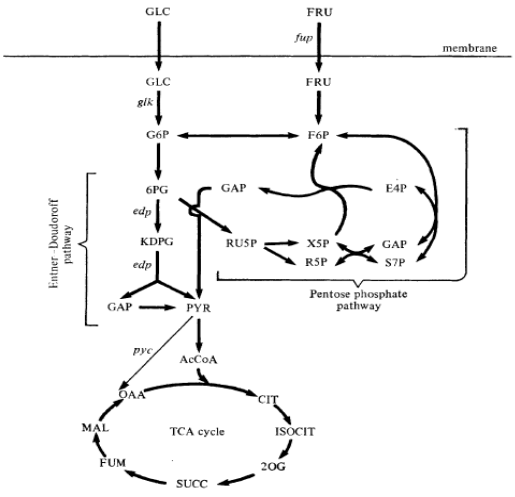

Fig. 1.14: Pathways of glucose and fructose catabolism available to *R. trifolii* strain 7000 (Ronson and Primrose, 1979). The mutants are blocked at the steps indicated: *glk,* strains 7009, 7013 and 7039; *fup,* strain 7039; pyc, strain 7049. Strain 7028 is blocked at one of the two steps labelled *edp*. Abbreviations: GLC, glucose; FRU, fructose; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 6PG, 6-phosphogIuconate; KDPG, 2-keto-3-deoxy-6-phosphogluconatGe;A P, glyceraldehydes 3-phosphate; RUSP, ribulose 5-phosphate; X5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; PYR, pyruvate; AcCoA, acetyl-CoA; OAA,

oxaloacetate; CIT, citrate; ISOCIT, isocitrate; 20G, 2-oxoglutarate; SUCC, succinate; FUM, fumarate; MAL, malate; TCA, tricarboxylic acid.

Rhizobium trifolii strain 7000 contained key enzyme activities of the ED and PP pathways (**Fig. 1.14**). The lack of phosphofructokinase indicated that the EMP pathway was absent (Ronson and Primrose, 1979).

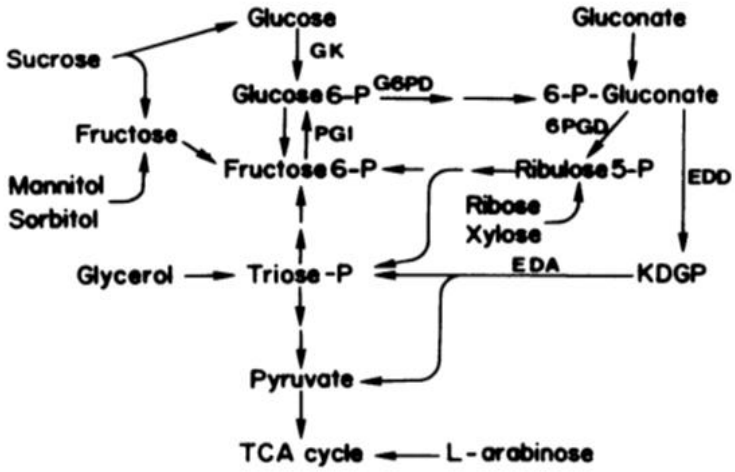

Fig. 1.15: Outline of Possible pathways of Carbohydrate metabolism in *R. meliloti* (Arias et al., 1979).

1.2.4: Direct oxidative pathway.

1.2.4.1: Gluconic and 2-ketogluconic acid secretion.

Phosphorous is second macronutrient required by plant for the growth and development, in soil phosphorous is present in form of (organic and inorganic forms) (Zou et al., 1992; Vance, 2001). Worldwide crop production is decreasing due to P deficiency (Arcand and Schneider, 2006). Phosphorous gets re fixed in large proportion and rapidly precipitated , in the form of Fe–P and Al–P complexes, which becomes unavailable to plants (Johnson and Loepper, 2006; Rengel and Marschner, 2005). As an alternative strategy, to make free P available to plants is the mineral phosphate solubilizing (MPS) by microorganisms. Microorganisms are known to solubilize mineral phosphate by secreting variety of low molecular weight organic acids such as gluconic, 2-ketogluconic, citric and oxalic.

In many Gram-negative bacteria's the MPS mechanism is well characterized, as it involves gluconic acid secretion by a direct oxidation pathway, which is mediated by the membrane-bound glucose dehydrogenase (GDH) (Kim et al., 1997; Liu et al., 2006; Patel et al. 2008). GDH enzyme requires Pyrroloquinoline quinone (PQQ) as a cofactor, for glucose oxidation to convert glucose to gluconic acid. Katznelson et al. (1962) provided first evidence of Direct Oxidative pathway in MPS. Gluconic acid in periplasmic space undergoes for oxidations to produce 2-ketogluconic acid which is mediated by gluconate dehydrogenase (GADH) (Anderson et al. 1985). Amongst the organic acids secreted into the extracellular medium by bacteria,gluconic and 2-keto gluconic acids are strongest (Duine 1991). These acids act as Ca^{2+}chelators under suitable conditions and attribute for acidification and make free P available, from calcium phosphates such as tri calcium phosphate (TCP) or hydroxyapatite (HAP) (Krishnaraj and Goldstein 2001).

Pseudomonads are significant due to their plant growth promoting and phosphate solubilizing abilities. Under P-limitation, due to compromised metabolic status, intracellular phosphorylative pathway of glucose oxidation in both the pseudomonads was subdued while enhanced direct oxidative pathway which could benefit the metabolic status since GDH activity is directly coupled to electron transfer and generation of proton motive force (van Schie et al, 1985). This may also explain why most of the rhizospheric MPS bacteria employ direct oxidation pathway mediated gluconic or 2-ketocgluconic acid secretion for P-solubilization. Compromised metabolic status of *P. fluorescens*13525 under P-deficient conditions was overcome in *Pseudomonas* P4 by shifting the metabolism towards direct oxidation pathway producing high gluconic acid levels which facilitated ATP generation as a consequence of improved Pi availability. Metabolic flexibility/rigidity behind gluconic acid secretion in P-solubilizing pseudomonads could facilitate metabolic engineering strategies for enhancing the MPS ability of *Pseudomonas* strains.

Pseudomonads catabolize glucose by two different routes: the direct oxidative pathway which acts on glucose extracellularly and the simultaneously operating intracellular phosphorylative pathway. Early glucose dissimilation studies showed that in most of the pseudomonads glucose oxidation occurs in two successive reactions forming D-gluconate and 2-keto-D-gluconate (2-KG) catalyzed by a membrane-bound PQQ-GDH and gluconate dehydrogenase (GADH) respectively, in the periplasm (Lessie and Phibbs,

1984). Since then direct oxidative pathway has been demonstrated to occur in *Pseudomonas fluorescens* 52-1C (Fuhrer et al., 2005), *P. putida* U (Schleissner et al., 1997), *P. putida*KT2442 (Basu and Phale, 2006) and *P. aeruginosa* 2F32 (Midgley and Dawes, 1973). Although the direct oxidation pathway was not found in *P. putida*KT2440 (Lessie and Phibbs, 1984; Fuhrer et al., 2005), its genome sequence showed the presence of the PQQ-GDH encoding *gcd* gene (Nelson et al., 2002).

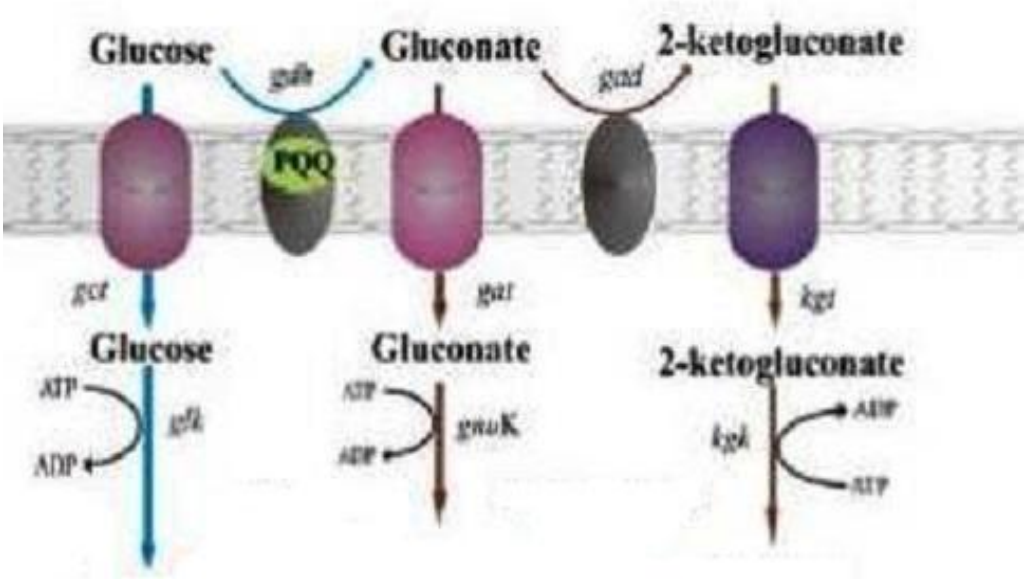

Fig. 1.16: Direct oxidative pathway in *Pseudomonads* (Archana et al., 2012).

1.2.4.2: Importance of Pyrroloquinoline quinone in abiotic stress.

Hauge (1964) predicted Pyrroloquinoline quinone (PQQ) as a bacterial cofactor which was confirmed as methoxatin by Salisbury (1980; 1981) **(Fig.1.17).** Under proper conditions, PQQ is water soluble and heat stable and can catalyze ~20000 redox cycling (continues oxidation and reduction reactions) events (Rucker et al., 2009) **(Fig.1.18).**

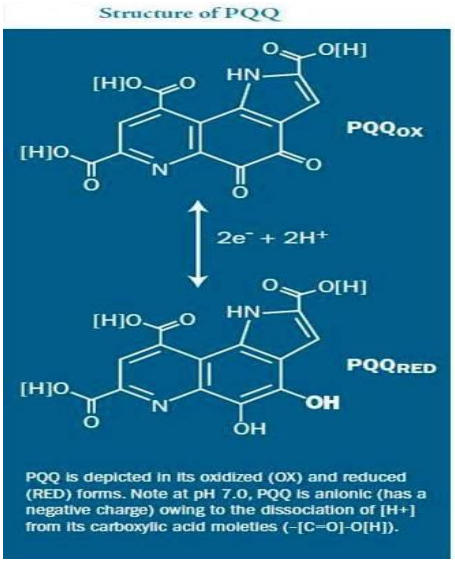

Fig. 1.17: Structure of PQQ (Rucker et al., 2009).

Compound	Potential Number of Catalytic Cycles
PQQ	20,000
Quercetin	800
Catechin	75
Epicatechin	700
Norepinephrine	200
Epinephrine	100
DOPA	20
6-OH-DOPA	20
Ascorbic Acid	4

Fig. 1.18: PQQ as a redox cycling agent (Rucker et al., 2009).

PQQ biosynthesis in bacteria involves varying number of genes present in clusters (**Fig. 1.19**) (Choi et al., 2008). PQQ biosynthesis is not completely understood but a putative biosynthetic pathway has been proposed on the basis of the functions of conserved genes in bacteria (Puehringer et al., 2008). *Klebsiella pneumonia* possesses *pqqABCDEF* genes which are involved in PQQ biosynthesis (Meulenberg et al., 1992). *pqqA* gene encodes for 23-24 amino acid polypeptide which a substrate for a set of enzymes modifying the glutamate and tyrosine residues leading to PQQ formation

(Goosen et al. 1992; Meulenberg et al. 1992; Velterop et al. 1995). PqqB belongs to metallo-β-lactamases and has been suggested to help in the transport of PQQ into periplasm (Velterop et al., 1995). PqqC is a cofactor less oxidase, activates oxygen, and catalyzes the final step of ring closure reaction (Magnusson et al., 2004). PqqD has been shown to interact with PqqE possesses reductively cleavage activity of S-adenosyl methionine to form 5' deoxyadenosine and methionine (Wecksler et al., 2010). PqqE catalyzes the first step of linking glutamate and tyrosine residues of PqqA peptide. Additionally, PqqF, G, H, I, J, K and M are found in some bacteria possessing putative Zn dependent peptidase, non-catalytic subunit of peptidase, transcriptional regulator, aminotransferase, cytosolic protein, DNA binding and prolyloligopeptidase, respectively (Choi et al., 2008).

Sr. No	**Genes**	**Function**
1	*pqqA*	18-22 amino acid peptide and serves as the precursor substrate for PQQ
2	*pqqB*	Carrier for PQQ and responsible for its transport across the plasma-membrane into the periplasm,
3	*pqqC*	Catalyzes final step of the PQQ biosynthesis,
4	*pqqD*	Interacts with PqqE and possesses reductive cleavage activity.
5	*pqqE*	Catalyzes the first step of linking glutamate and tyrosine residues of PqqA peptide.
6	*pqqF*	Zn dependent peptidase,
7	*pqqH*	Transcriptional regulator, LysR family
8	*pqqI*	Aminotransferase
9	*pqqJ*	Putative cytoplasmic protein
10	*pqqK*	Probable DNA-binding protein
11	*pqqM*	Peptidase

Table 1.5: Functions of *pqq* genes (Choi et al., 2008; Puehringer et al., 2008; Wecksler et al., 2010).

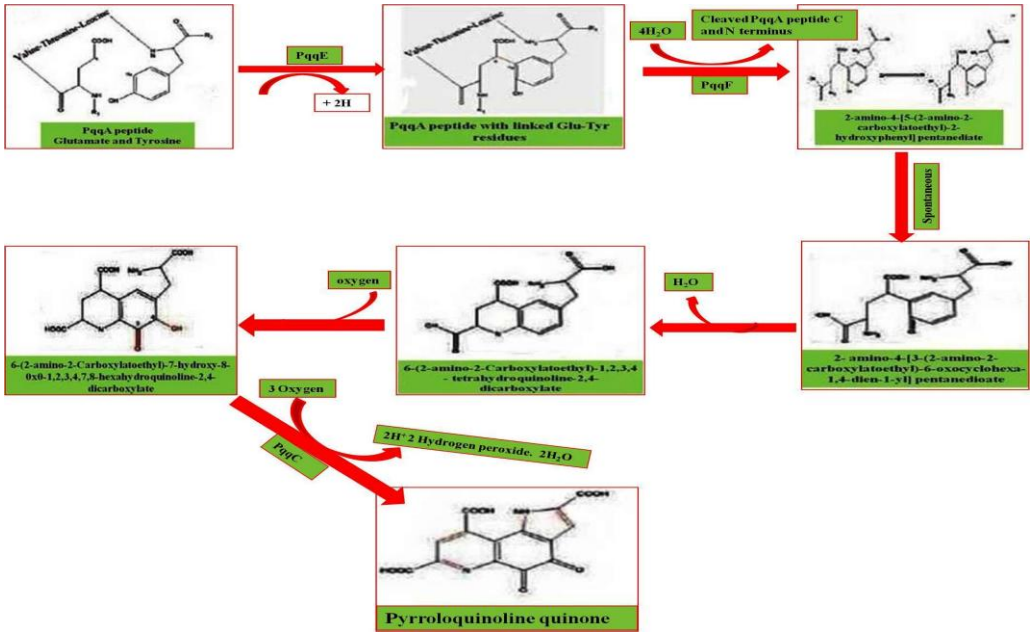

Fig. 1.19: PQQ biosynthesis pathway (Schwarzenbacher et al., 2008; Caspi, 2010).

PGPR promote plant growth which is mediated by antibiotics or siderophores, phytohormones production, inhibition of pathogenic microorganisms and MPS in soil (Leong, 1986; Sivan and Chet, 1992;Xie et al., 1996; De Freitas et al., 1997). PQQ has been found in various foods in nanogram range but plants and animals do not produce PQQ (Kumazawa et al., 1992, 1995; Choi et al., 2008). PQQ directly neutralizes reactive oxygen species (ROS) (Misra et al., 2004). PQQ – GDH are involved in production of anti-pathogen compounds (James and Gutterson 1986; Schnider et al. 1995; Han et al. 2008; de Werra et al. 2009; Guo et al. 2009; Ahmed and Shahab, 2010).

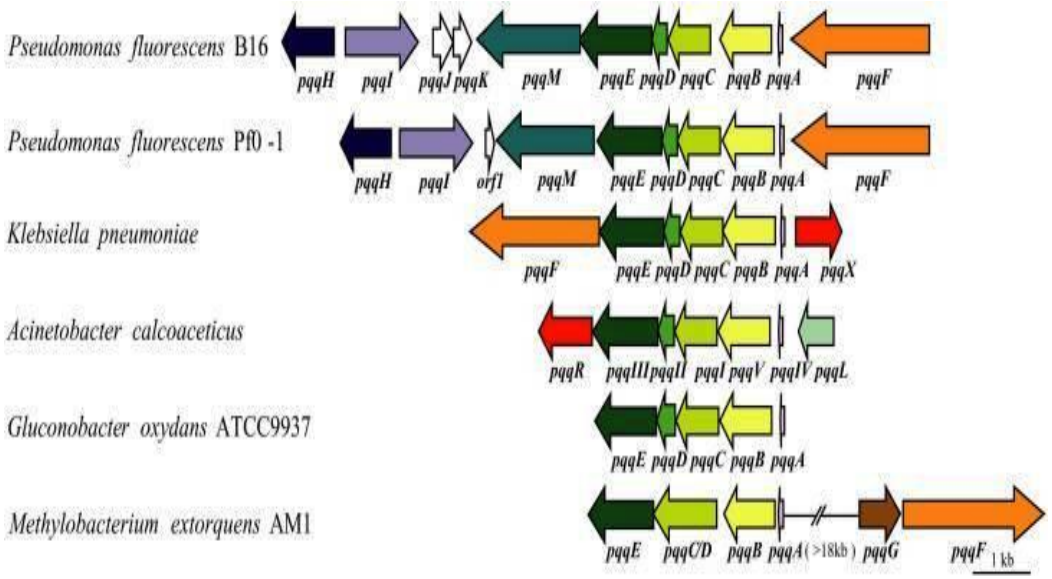

Fig. 1.20 : Comparison of *pqq* gene clusters of *P. fluorescens* B16, *P. fluorescens* Pf0-1, *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus*, *Gluconobacter oxydans* ATCC9937 and *Methylobacterium extorquens* AM1 (Choi et al., 2008).

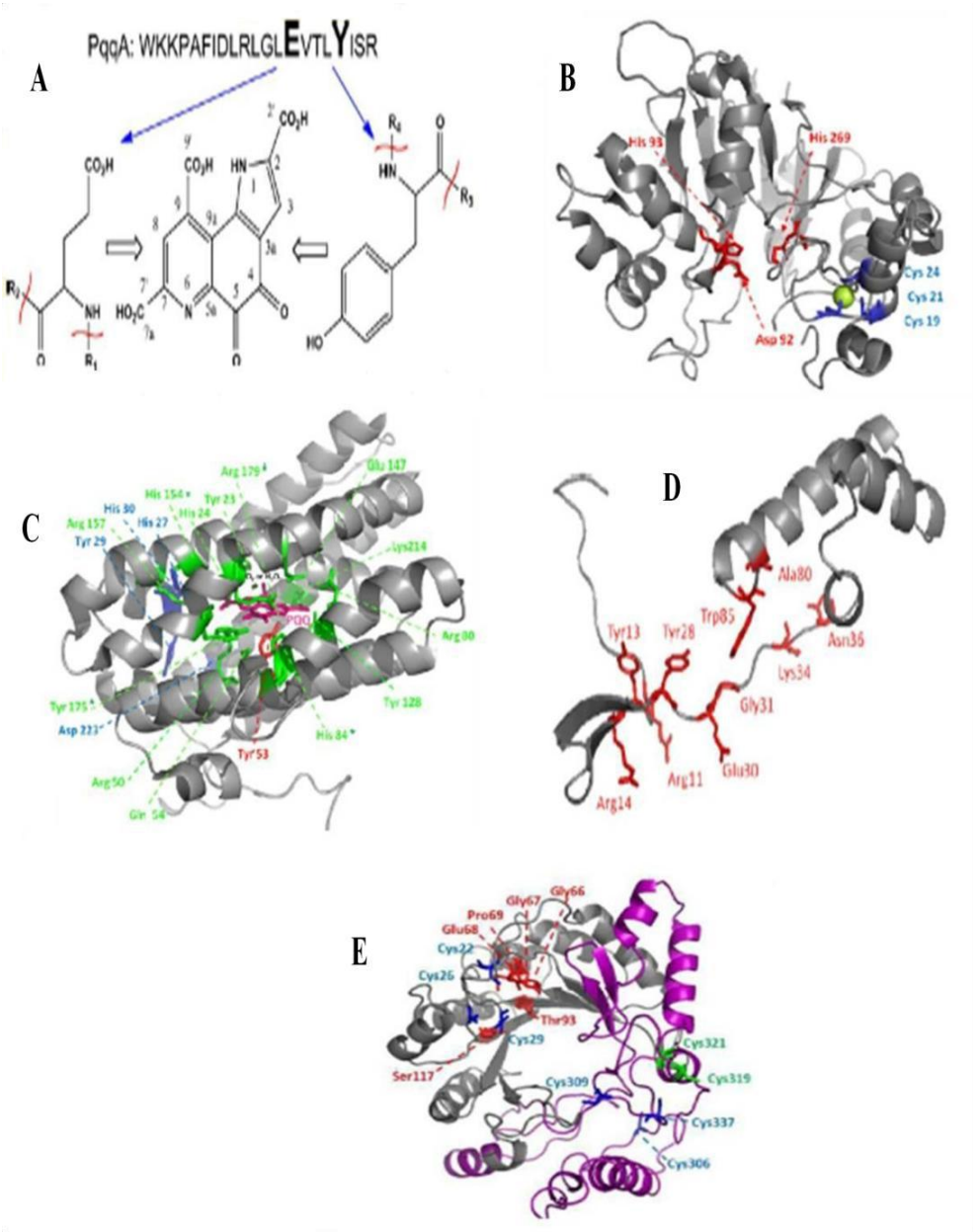

Fig. 1.21: Structures of Pqq genes. A- PqqA, B- PqqB, C- PqqC, D- PqqD and E- PqqE (Shen et al., 2012).

Rationale of Study

Microorganisms as biofertilizers assist plants to grow by increasing the quantity of nutrients. The living microorganisms that co-exist with the plants, promote the supply of important nutrients and, consequently are crucial for the overall productivity of the soil. Using biofertilizers offers a better option in reducing agrochemical inputs and maintain soil fertility and strength. Various free living soil bacteria that are capable of exerting beneficial effects on plants and can lead to increased yields of a wide variety of crops, are known as plant growth promoting rhizobacteria (PGPR). PGPR can promote growth by various mechanisms like production of phytohormones, asymbiotic nitrogen fixation, solubilization of mineral phosphates and other nutrients and antagonism against phytopathogens by production of siderophores, chitinases, antibiotics, and by lowering endogenous levels of plant hormone ethylene in roots. PGPR strains may use one or more of these mechanisms in the rhizosphere.

There is a mutualistic symbiotic association between legumes and rhizobia, which results in the formation of nodules (nitrogen fixing sites) on the roots of legumes. Seed inoculation of pulse crops with effective Rhizobial strains prior to sowing is a recommended practice, as it improves nodulation and N-fixation, which in turn is translated into enhanced growth and grain yield. Phosphorus is an essential ingredient for *Rhizobia* to convert atmospheric N (N_2) into an ammonium (NH_3) form usable by plants. Phosphorus becomes involved as an energy source for nitrogen fixation. Phosphorus influences nodule development. Inadequate P restricts root growth, the process of photosynthesis, translocation of sugars, and other such functions which directly or indirectly influence N fixation by legume plants. Phosphorus (P) is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available. Efficiency of P fertilizer throughout the world is around 10-25 %, and concentration of bio-available P in soil is very low reaching the level of 1.0 mg kg^{-1} soil. Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants. Inorganic forms of P are solubilized by a group of heterotrophic microrganisms excreting organic acids that dissolve phosphatic minerals and / or chelate cationic partners of the P ions directly, releasing P into solution.

Direct periplasmic oxidation of glucose to gluconic acid is considered as the metabolic basis of inorganic phosphate solubilization by many Gram-negative bacteria as a competitive strategy to transform the readily available carbon sources into less readily utilizable products by other microorganisms Gluconic acid is the principal organic acid produced by *Pseudomonas* spp., *Erwinia herbicola*, *Bacillus* spp., *Burkholderia* spp., *Rhizobium* spp. Other organic acids such as lactic, isovaleric, isobutyric, acitic, glycolic, oxalic, malonic and succinic acids are also generated by the different phosphate solubilizing bacteria.

Researchers have demonstrated that an efficient mineral phosphate solubilizing phenotype in Gram-negative bacteria resulted from extracellular oxidation of glucose to gluconic acid *via* Quinoprotein glucose dehydrogenase equipped with Pyrroloquinoline quinone (PQQ) as a cofactor and gluconic acid oxidation to 2-ketogluconic takes place *via* the FAD linked gluconate dehydrogenase (GADH) (Buurman et al.1994; Buch et al. 2008). Both enzymes are in the outer face of the cytoplasmic membrane, thus acids are formed in the periplasmic space, with the resultant acidification of this region and, ultimately, the adjacent medium as well. Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization, along with 2-ketogluconic acid as another organic acid identified in strains with phosphate solubilizing ability.

Carbohydrate metabolism in *Rhizobium* is different from that of model microorganisms, *E. coli* and *Pseudomonas*. In *E. coli*, glycolysis and tricarboxylic acid (TCA) cycle are the main catabolic pathways for sugars while Entner Duodroff pathway, pentose phosphate pathway and TCA cycle operate in *Pseudomonas*. Along with these direct oxidation pathway of glucose to gluconic acid, 2-ketogluconic acid is observed in *Pseudomonas*. Carbohydrate metabolism in *Rhizobium* is similar to *Pseudomonas* but differs in its capacity for direct oxidation of glucose to gluconic acid and 2-ketogluconic acid. Some species of *Rhizobium* have PQQ dependant Glucose dehydrogenase (apo-GDH) or Gluconate dehydrogenase (GADH) present but the enzyme PQQ synthase which is required for GDH is absent thus does not allow glucose oxidation in the periplasm.

Genetic manipulation by recombinant DNA technology offers a feasible approach for obtaining improved strains. Cloning of genes involve in mineral phosphate solubilization (mps), such as those influencing the synthesis of gluconic and 2-ketogluconic acids would be the first step in such genetic manipulation program. Since, some species of

rhizosphere competent *Rhizobium* possess apo-GDH and/or GADH, it would be interesting to transfer the genes involved in PQQ biosynthesis, GDH and GADH to strains of *Rhizobium* which lack these enzymes and transform *Rhizobium* into a PSM.

Immunological and molecular techniques have been used to quantify and identify inoculated strains on plant roots (Benizri et al., 2001). Immunological techniques such as ELISA and immunofluorescence colony staining have helped in quantification and visualization of the strain on the plant roots. Molecular techniques also aid in quantification and visualization. Tagging bacteria with marker or reporter genes facilitates identification of the strain on the plant roots. Colorimetry is used for detection of gene products of *lacZ* and *xylE* (Benizri et al. 2001). Other markers, which are observed with the help of charge-couple device cameras and confocal laser scanning microscope (CLSM), are the Lux (luciferase) and GFP (green fluorescent protein) gene products.

Aequorea victoria Green fluorescent Protein (GFP) and its variants and homologs of different colors are used in a variety of applications to study the organization and function of living systems , to observe localization, movement, turnover, and even "aging" (i.e., time passed from protein synthesis). GFPs targeted to cell organelles by specific protein localization signals enable visualization of their morphology, fusion and fission, segregation during cell division, etc. GFPs are essential tools for individual cell labeling and tissue labeling to visualize morphology, location, and movement (e.g., during embryonic development and tumorigenesis), mitotic stages, and many other important cell characteristics. Finally, whole organisms can be labeled with GFPs to discriminate between transgenic and wild-type individuals (Lukyanov et al., 2010).

Enhanced green fluorescent protein in conjunction with CSLM has been used in studying the pattern of root colonization by strain WCS365 on tomato roots (Lugtenberg et al., 2001). *Azospirillum*-wheat interactions were monitored using the *gfp* and *gusA* genes (Pedrosa et al., 2002). Charcoal based bioformulation of *Rhizobium* species using GFP showed enhanced growth and root colonization in *Cajanus cajan* (Maheshwari et al., 2009). Studies on Colonization of sugarcane and rice plants by the endophytic diazotrophic bacterium *Gluconacetobacter diazotrophicus* were studied using *gfp* and *gusA* reporter genes (Schwab. et al., 2010). The use of GFP fluorescently tagged bacteria and CLSM demonstrated that *P. fluorescens* PICF7 effectively colonizes roots in invitro propagated olive plants under gnotobiotic conditions (González et al., 2011).

Vitreoscilla sp. hemoglobin (VHb) is an oxygen-binding protein, heterologous expression of *vgb* gene in recombinant bacterium shows an increase in chemical energy content, and expression of the nitrogen fixation gene *nifH*. Plants inoculated with the engineered *Rhizobium* strain showed significantly enhanced nitrogenase activity and total nitrogen content compared with plants inoculated with the wild-type strain (Ramírez et al., 1999). VHb synthesis stimulated the respiratory efficiency of free-living rhizobia as well as of symbiotic bacteroids leading to higher levels of nitrogen fixation and improves symbiotic performance.

The bacterial chromosome may be used to stably maintain foreign DNA in the mega-base range. Integration into the chromosome circumvents issues such as plasmid replication, plasmid stability, plasmid incompatibility, and plasmid copy number variance. Stable integration of expression cassettes into bacterial chromosomes would prevent the need of antibiotic selection for the expression of recombinant proteins. An integration system, known as the Tn*7*-based broad-range bacterial cloning and expression system integrates recombinant DNA fragments into a specific site on the bacterial chromosome, known as the *att*Tn*7* site. The *att*Tn*7* site has been localized in the intergenic region in several gram-negative bacteria, including, notably, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *P. putida*, *Rhizobium* and *Yersinia pestis*. The Tn*7* system does not cause insertional inactivation of host genes and therefore permits construction of isogenic strains that differ only in the nature of the added DNA (Lambertsen, 2004).

This transgenic *Rhizobium* having P solubilizing activity could be effective as N and P biofertilizer. Thus the objective of the present study involves:

1. Determining the MPS ability of *Rhizobium* strains (*B. japonicum* and *M. loti*) by incorporating *pqqE* gene and *Acinetobacter calcoaceticus pqq* gene cluster.
2. Determining the MPS ability of *Rhizobium* strains containing *pqq* cluster and *Pseudomonas putida gad* operon.
3. Genomic integration of *pqq* cluster and *gad* operon with *vhb-gfp* in *Rhizobium* strains and determining the MPS ability.
4. Plant growth promotion study of integrants.

Chapter 2

Materials and methods

2: Materials and Methods

2.1 Bacterial strains / Plasmids

All the plasmids use in the present study and their restriction maps are given in **Table 2.1, Fig. 2. 1** The wild type and genetically modified *E. coli* and *Rhizobium* strains (*Bradyrhizobium japonicum* USDA110; *Mesorhizobium loti* MAFF303099 and *Sinorhizobium freddi* (NGR234) are listed in **Table 2.1** *E. coli* DH10B was used for all the standard molecular biology experiments wherever required. *Rhizobium* strains used in all this study is generous gift from (Dr Poole, U.K. John Innes Center). The *pqqE* gene was gifted by Goldstein Department of Biology, California State University at Los Angeles, State University Drive, Los Angeles, California. *pqq* gene cluster of *Acinetobacter calcoaceticus* was gifted by (Dr. Goosen, Molecular Genetcis, University of Leiden, Netherland).

Table 2.1: List of plasmids used in the present study.

Plasmid/Bacterial strains	**Characteristics**	**Source/Reference**
E. coli DH10B	Used to maintain plasmids for routine use	Invitrogen, USA
pBBR1MCS-2	Broad-Host-Range vector; Km^r	Kovach et al., 1995
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r (100µg/ml)	Hester et al., 2000
pJNK1	pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Wagh, 2013
pJNK5	pUCPM18, Gm^r (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	Wagh, 2013
***Rhizobium* strains**		
Bradyrhizobium japonicum USDA110	NC_004463.1	NCBI
Mesorhizobium loti **MAFF030669**	NC_002678.2	NCBI
Sinorhizobium fredii **NGR234**	NC_012587.1	NCBI
***Bj* pUCPM18**	*B. japonicum* with pUCPM18, Gm^r (control vector)	Chapter 3
***Bj* pJNK1**	*B. japonicum* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Chapter 3
***Bj* pJNK5**	*B. japonicum* pUCPM18, Gm^r (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	Chapter 3
***Ml* pUCPM18**	*M. loti* with pUCPM18, Gm^r (control vector)	Chapter 3
***Ml* pJNK1**	*M. loti* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Chapter 3
***Ml* pJNK5**	*M. loti* with pUCPM18, Gm^r (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	Chapter 3
***Sf* pUCPM18**	*S. fredii* with pUCPM18, Gm^r (control vector)	Chapter 3
***Sf* pJNK1**	*S. fredii* with pBBR1MCS-2 Km^r with 1.8 kb *E. herbicola pqqE* gene	Chapter 3
***Sf* pJNK5**	*S. fredii with* pUCPM18, Gm^r (20µg/ml) with 5.1 Kb *pqq* gene cluster of *A. calcoaceticus*.	Chapter 3
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r (100µg/ml)	Hester et al., 2000
pJNK6	pJNK5, Gm^r (20µg/ml) with *gad* operon 3.8 Kb of *P. putida* KT 2440	Wagh, 2013
***Bj* pUCPM18**	*B. japonicum* with pUCPM18, Gm^r (control vector)	Chapter 4

***Bj* pJNK6**	*B. japonicum* with pJNK6Gm^r (20µg/ml)	Chapter 4
***Ml* pUCPM18**	*M. loti* with pUCPM18, Gm^r (control vector)	Chapter 4
***Ml* pJNK6**	*M. loti* with pJNK6Gm^r (20µg/ml)	Chapter 4
***Sf* pUCPM18**	*S. fredii* with pUCPM18, Gm^r (control vector)	Chapter 4
***Sf* pJNK6**	*S. fredii* with pJNK6Gm^r (20µg/ml)	Chapter 4
pGRG36	Intergration vector (Tn7)	McKenzie et al., 2006
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
E. coli DH10B	*Str^r F$^-$ endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZ ΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ$^-$*	(Invitrogen) USA
pJNK6	pUCPM18Gm^r with *pqq* gene cluster of *Acinetobacter calcoaceticus* and *gad* operon of *P. putida* KT2440 under *plac*; Ap^r, Gm^r	Wagh, 2013
pJIPgv	pGRG36 with *pqq* gene cluster of *Acinetobacter calcoaceticus* and *gad* operon of *P. putida* KT2440 under *plac*; Ap^r, *vgb* gene and *egf* gene Ap^r	Chapter 5
Bj intPgv	Genomic integrant of *B. japonicum* containing *lac-pqq,gad, vgb, egfp* Ap^r	Chapter 5
Ml intPgv	Genomic integrant of *M. loti* containing *lac-pqq,gad, vgb, egfp* Ap^r	Chapter 5
Sf intPgv	Genomic integrant of *S. fredii* containing *lac-pqq,gad, vgb, egfp* Ap^r	Chapter 5

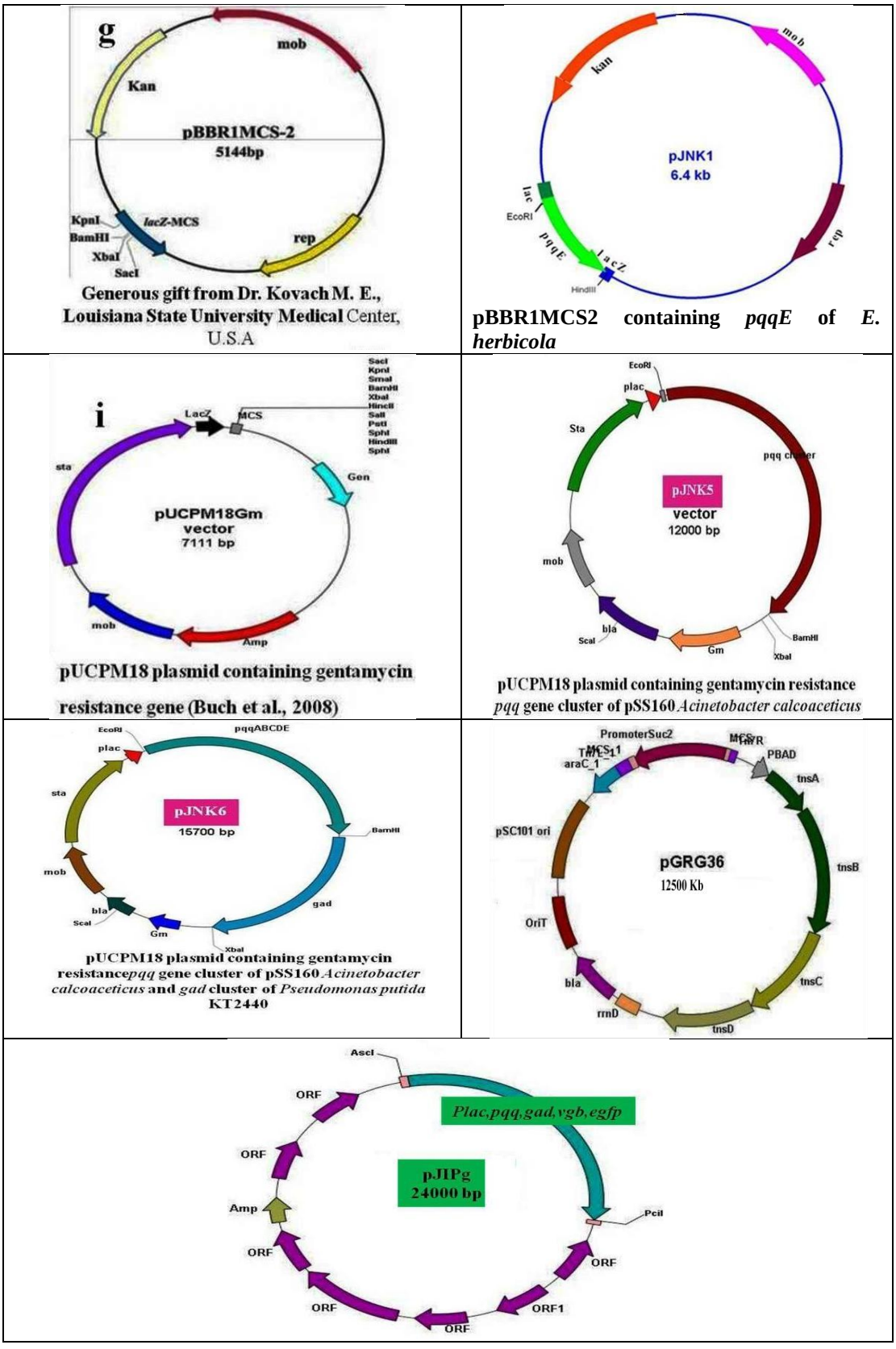

Fig. 2.1: Restriction maps of the plasmids used in this study. All plasmids are broad host- range vectors used for expression of heterologous overepression of genes under *lac* promoter in *Rhizobium* strains (*Bradyrhizobium japonicum* USDA 110; *Mesorhizobium loti* MAFF303099 and *Sinorhizobium freddi* (NGR234)

2.1.1: pUCPM18 plasmid is derived from a pUC-derivative, pUCP18 plasmid which is stably maintained in both *E. coli* and *Pseudomonas* species (Schweizer, 1991). pUCPM18 was developed from pUCP18 (GenBank accession number: U07164) by incorporating a 750bp mob fragment from pLAFRI (broad host range vector used for genetic analysis of gram-negative bacteria; Vanbleu et al., 2004), in order to enable convenient mobilization within *Pseudomonas* species (Hester et al, 2000). It replicates in *E. coli* using ColE1 origin of replication (*ori*) while in *Pseudomonas* it replicates owing to a pRO1614 derived DNA fragment encoding a putative *ori* and a replication-controlling protein (West et al., 1994).

2.1.2: pBBR1MCS-2 (GenBank accession number: U23751) plasmid conferring kanamycin resistance is originally derived from pBBR1 plasmid of *Bordetella bronchiseptica* (Kovach et al., 1995). This plasmid facilitates cloning due to availability of unique restriction sites and direct selection of recombinant plasmids based on blue white selection strategy. pBBR1MCS based vectors are mobilizable when the RK2 transfer functions are provided in *trans*, are compatible with IncP, IncQ and IncW group plasmids, as well as with ColE1- and P15a-based replicons, can stably replicate in a variety of hosts including *E. coli*, *P. fluorescens*, *P. putida*, *Rhizobium meliloti* and *R.leguminosarum bv. Viciae* and are stably retained *in vivo* for more than 4 weeks in absence of antibiotic selection (Kovach et al., 1995).

2.2: Media and Culture conditions

The *E. coli* strains and pseudomonads were cultured and maintained on Luria Agar (LA) and Pseudomonas Agar respectively (Hi-Media Laboratories, India). *E. coli* cultures were grown at 37ºC while all *Rhizobium* strains (*Bradyrhizobium japonicum* USDA 110; *Mesorhizobium loti* MAFF303099 and *Sinorhizobium freddi* (NGR234) cultures were grown at 30ºC. For growth in liquid medium, shaking was provided at the speed of 200rpm. The plasmid transformants of both *E. coli* and pseudomonads were maintained using respective antibiotics at the final concentrations as mentioned in (**Table 2.2)** as and when applicable. Both *E. coli* and *Rhizobium* strains (*Bradyrhizobium japonicum* USDA 110; *Mesorhizobium loti* MAFF303099 and *Sinorhizobium freddi* (NGR234) wild type strains and plasmid transformants

grown in 3ml Luria broth (LB) containing appropriate antibiotics were used to prepare glycerol stocks which were stored at -20ºC.

Antibiotic	**Rich medium**	**Minimal medium**
Nalidix acid	15 µg/ml	3 µg/ml
Gentamycin*	20µg/ml	5 µg/ml
Tetracycline	30µg/ml	7.5µg/ml
Kanamycin	50µg/ml	12.5µg/ml
Streptomycin	10µg/ml	2.5µg/ml
Trimethoprim	60µg/ml	15µg/ml
Ampicillin*	50µg/ml	12.5µg/ml
Erythromycin	100µg/ml	-
Chloramphenicol*	20µg/ml	-
Spectinomycin	50µg/ml	-

The compositions of different minimal media used in this study are as described below. Antibiotic concentrations in all the following minimal media were reduced to 1/4th of that used in the above mentioned rich media (**Table 2.2**).

Table 2.2: Recommended doses of antibiotics used in this study (Sambrook and Russell, 2001). The antibiotic dozes were maintained same for both *E. coli* and pseudomonads. All the antibiotics were prepared in sterile distilled water or recommended solvent at the stock concentrations of 1000x or 2000X (for antibiotics marked with *) and were used accordingly to have the desired final concentrations.

2.2.1: M9 minimal medium

Composition of M9 minimal broth was according to Sambrook and Russell (2001) including Na_2HPO_4 $7H_2O$, 34g/L; KH_2PO_4, 15g/L; NH_4Cl, 5g/L; NaCl, 2.5g/L; 2mM $MgSO_4$; 0.1mM $CaCl_2$ and micronutrient cocktail. The micronutrient cocktail was constituted of $FeSO_4.7H_2O$, 3.5 mg/L; $ZnSO_4.7H_2O$, 0.16 mg/L; $CuSO_4.5H_2O$, 0.08 mg/L; H_3BO_3, 0.5 mg/L; $CaCl_2.2H_2O$, 0.03 mg/L and $MnSO_4.4H_2O$, 0.4 mg/L. Carbon sources used were glucose, xylose, fructose and lactose as and when required. For solid media, 15g/L agar was added in addition to above constituents. 5X M9 salts, micronutrients (prepared at 1000X stock

concentration) and carbon source (2M stock) were autoclaved separately. Fixed volumes of these were added aseptically into pre-autoclaved flasks containing distilled water to constitute the complete media with the desired final concentrations. Volume of water to be autoclaved per flask was calculated by subtracting the required volumes of each ingredient from the total volume of the media to be used.

2.2.2: TrisCl buffered medium

The media composition included TrisCl (pH=8.0), 100 mM; NH_4Cl, 10 mM; KCl,10 mM; $MgSO_4$, 2 mM; $CaCl_2$, 0.1 mM; micronutrient cocktail; Glucose, 100mM and phosphate (P) sources (Sharma et al., 2005). 1mg/ml Senegal Rock phosphate (RP) or KH_2PO_4 were used as insoluble and soluble P sources respectively. Each ingredient was separately autoclaved at a particular stock concentration and a fixed volume of each was added to pre-autoclaved flasks containing sterile distilled water (prepared as in Section 2.2.3) to constitute complete media.

2.2.3: Pikovskaya's (PVK) Agar

The media composition included Ammonium sulphate, 0.5g/L; Calcium phosphate, 5.0g/L; Dextrose, 10.0g/L; Ferrrous sulphate, 0.0001g/L; Magnesium Engineering the glucose metabolism of *Rhizobium* spp. by heterologous expression of *pqq* gene cluster, *gad* operon and *vgb* gene sulphate, 0.1g/L; Manganese sulphate, 0.0001g/L; Potassium chloride, 0.2g/L; Yeast extract, 0.5g/L and agar, 15.0g/L. Dextrose was substituted by same amount of xylose and fructose as and when mentioned. The readymade media was obtained from Hi-Media Laboratories, India, and was used according to manufacturer's instructions.

2.2.4: Murashige-Skoog's Medium

Murashige-Skoog's (MS) medium was composed of macro elements and micro elements. The macro elements included $CaCl_2.2H_20$, 0.440g/L; KH_2PO_4, 0.17g/L; KNO_3, 1.9g/L; $MgSO_4.7H_2O$, 0.37g/L; NH_4NO_3, 1.65g/L and 10g/L agar. The micro elements included the essential trace elements. Micronutrients and desired carbon source (autoclaved separately), were added aseptically to reconstitute the complete media. The readymade micronutrient was obtained from Hi-Media

Laboratories, India, and was used according to manufacturer's instructions while the macronutrients were reconstituted as and when required.

2.2.5: YEMA medium for *Rhizobium* strains

Yeast Mannitol Agar with congo red was used to maintain *Rhizobium* species on plates, YEMA medium contains ingredients gms/ L. Yeast extract 1.000, Mannitol 10.000, Dipotassium phosphate 0.500, Magnesium sulphate 0.200, Sodium chloride 0.100, Congo red 0.025, Agar 20.000, Final pH (at 25°C) 6.8±0.2 (Vincent, J.M. 1970).

2.2.6 Tryptone Yeast Extract Medium (TYE)

TY medium contained **0.5** % (w/v) Difco Bacto-Tryptone, 0.3 % (w/v) Difco Bacto-Yeast Extract and 7 mM-CaC1, Liquid PA medium contained 0.4 % (w/v) Difco Bacto-Peptone and **2** mM-$MgSO_4$, (Hirsch et al., 1980)

2.3: Molecular biology tools and techniques

2.3.1: Isolation of plasmid and genomic DNA

2.3.1.1: Plasmid DNA isolation from *E. coli* and *Rhizobium* strains.

The plasmid DNA from *E. coli* and *Rhizobium* was isolated by the boiling alkali lysis method, (Sambrook and Russell, 2001). Engineering the glucose metabolism of *Rhizobium* strains by heterologous expression of and *pqq* gene clusters and *gad* operon.

2.3.1.2: Genomic DNA from *Rhizobium* strains.

The plasmid DNA from *E. coli* and *Rhizobium* strains was isolated by the method, (Sambrook and Russell, 2001).

2.3.2: Transformation of plasmid DNA

2.3.2.1: Transformation of plasmid DNA in *E. coli*

The transformation of plasmids in *E. coli* using $MgCl_2$-$CaCl_2$ method and blue white selection of the transformants using IPTG and X-Gal (as and when applicable) was carried out according to Sambrook and Russell (2001).

2.3.2.2: Electroporation of plasmid DNA in *Rhizobium* strains.

Plasmid transformation in *Rhizobium* strains was done by electroporation as described by (Unge et al., 1998) *Rhizobium* strains cells grown till early stationary phase, cells were harvested (at 4^oC) in sorvall washed 4 times with sterile distilled water and resuspended at 10^{10} cells ml^{-1}. Plasmid DNA of 0.5 µg $µl^{-1}$ was added in competent cells in electroporation cuvette. After 10 min of incubation on ice, the DNA was electroporated into cells using Eppendorf Electroporator system. System was adjusted to 2.5 kV ans 186 Ω. The cells were immediately transformed to 1 ml LB and incubated for 45 min at 30^oC, and then cells were plated on respective antibiotic for the selection of plasmid.

2.3.3: Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% agarose (containing 1µg/ml ethidium bromide) gel in Tris-acetate-EDTA (TAE) buffer at 5v/cm for 0.5-2h. The DNA bands were visualized by fluorescence under the UV-light using UV transilluminator.

2.3.4: Restriction enzyme digestion analysis

0.5-1.0µg DNA sample was used for each restriction enzyme digestion. 1-3U of the restriction endonuclease (RE) was used with the appropriate 10X buffers supplied by the manufacturer in a final reaction volume of 10µl. The reaction mixture was incubated overnight at 37°C. The DNA fragments were visualized by ethidium bromide staining after electrophoresis on 0.8% agarose gels and were subsequently

photographed. In case of double digestion, a compatible buffer for the two REs was essentially checked. If not available, digestion with one enzyme is performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

2.3.5: Gel elution and purification

The DNA fragments of desired sizes were recovered from the gel by cutting the agarose gel slab around the DNA band. The agarose piece was weighed in a sterile microcentrifuge tube and was solubilized in 2.5 volumes of 6M sodium iodide (NaI, freshly made) [e.g. for 200mg of agarose piece, 500µl of NaI was added]. Once completely dissolved, 15µl of silicon dioxide suspension (50% w/v, stored at 4°C) was added and was incubated at room temperature for 15-20 minutes. The DNA bound to silica was recovered by centrifuging at 9, 200x *g* for 2 minutes; the pellet was washed twice with 70% ethanol, dried, and finally re-suspended in 20-30µl sterile double distilled water. The microcentrifuge tube was incubated at 55°C for 10 minutes to allow complete dissociation of DNA from silica beads into the solution and then was subjected to centrifugation at 9,200x *g* for 2 minutes. The resultant supernatant was gently recovered using sterile micropipette tip and was transferred to fresh sterile tube. The purification efficiency was checked by subjecting 2µl DNA solution to gel electrophoresis and visualizing the sharp DNA band of desired size. The purified DNA was used for ligation experiments only if >50ng/µl DNA was recovered after purification.

2.3.6: Ligation

The ligation reaction was usually done in 10µl volume containing the following constituents: Purified vector and insert DNA (volume varied depending on the respective concentrations); 10X T4 DNA Ligase buffer, 1µl; T4 DNA ligase (MBI Fermentas), 0.5- 1.0U and sterile double distilled water to make up the volume. The cohesive end ligation reaction was carried out at 16°C for 12-16h. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:4 was maintained, with a total of 50-100ng of DNA in each ligation system.

$$\text{pmoles of DNA} = \frac{\text{Amount of DNA } (\mu g) \times 1,515}{\text{Size of the DNA fragment (no. of base pairs)}}$$

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2.3.7: Polymerase Chain Reaction (PCR)

The PCR reaction set up was based on the guidelines given in Roche Laboratory Manual. The assay system and the temperature profile used are described in **Table 2.6.**

Assay system used		**Temperature Profile**	
Sterile DDW	38 µl	Initial denaturation	94°C- 5 min
dNTP(10mM with 2.5mM each)	3 µl	Denaturation	94°C- 30 sec
Reverse Primer 20pmoles	1 µl	Annealing	Varies from 55-62°C for 30sec.*
Forward Primer 20pmoles	1 µl	Elongation	72°C for 45sec-2.5min*
Template DNA (100ng/µl)	1 µl		
Taq/20A PCR buffer (10X)	5 µl	Final Elongation	72°C- 10 min
Taq DNA/XT20 Polymerase (1 unit/µl)#	1.0 µl	For plasmid	(30 cycles)
Total System	**50 µl**	For genomic DNA	(40 cycles)

Table 2.3: PCR conditions used in the present study.

PCR amplifications were performed in Techne TC-312 thermal cycler. *Exact primer annealing temperature and primer extension time varied with primers (designed with respect to different templates) and has been specified in the text as and when applicable. Processivity of Taq polymerase is ~1000 bases per min. Taq DNA polymerase and its buffer, dNTPs and primers were obtained respectively from Bangalore Genei Pvt. Ltd., India, Sigma Chemicals Pvt. Ltd. and MWG Biotech. Pvt.

Ltd, India, respectively and were used according to manufacturer's instructions. The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the appropriate %G-C was carried out with the help of online primer designing software Primer 3. The sequence, length, and %G-C content of primers are subject to variation depending on the purpose of PCR and will be given as and when applicable in the following chapters. The PCR products were analyzed on 1.0% agarose gel along with appropriate molecular weight markers (Section 2.4.4)

2.3.8: Genomic Integration.

Integration of genes in genome was done by using miniTn7 based transposon integration vector, which is temperature sensitive. After electroporation of plasmid in Rhizobium strains culture was allowed to grown on YEMA plate and kept at 42^{o}C for 24 h for integration of genes at *att* sites of genome. (Nancy et al., 2006) confirmation of integrants was done by PCR amplification from genomic DNA.

2.4: P-solubilization phenotype

Fresh culture of *Rhizobium* strains transformants obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30ºC was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed thrice with sterile normal saline and used to characterize P-solubilizing ability of the native as well as the *H. seropedicae* transformantswere monitored for (i) di-calcium phosphate (DCP) solubilziation on Pikovaskya's (PVK) agar (Pikovskaya, 1948),(ii) TrisCl buffered RP-Methyl red as pH indicator dye and 1.5% agar (TRP) agar plates with addition of yeast extract 0.1% (Gyaneshwar et al., 1998). Culture was aseptically spotted on the TRP agar plates and was allowed to dry completely followed by incubation at 30°C for 2-4 days. P solubilization was determined by monitoring the zone of clearance on PVK agar and red zone on the TRP Methyl red agar plates.

2.5: Physiological experiments

The physiological experiments were carried out using various WT and transformant *Rhizobium* strains which included growth, pH profile and enzyme assays.

2.5.1: Inoculum preparation

The inoculum for M9 and HEPES minimal media containing rock phosphate were prepared by growing the *Rhizobium* strains overnight at 30°C in 3ml LB broth. Inoculum for the buffered RP broth (HRP) was prepared by growing the *Rhizobium* strains overnight at 30°C in 10ml of M9 minimal medium. Cells were harvested aseptically, washed twice by normal saline, and finally re-suspended in 1ml normal saline under sterile conditions. Freshly prepared inoculum was used for all the experiments.

2.5.2: Growth characteristics and pH profile

Growth parameters and pH profile of the native as well as *Rhizobium* strains transformants were determined using TRP medium with RP as P source (Gyaneshwar et al., 1998). Growth curve and pH profile was carried in 150ml conical flasks containing 30ml of relevant media. The initial O.D.600nm was about 0.09-0.15. The batch culture studies were performed under aerobic conditions in Orbitek rotary shaker maintained at 30°C with agitation speed kept constant at 200 rpm. 1ml samples were aseptically harvested at regular intervals and were subjected to analytical techniques. Microaerobic condition was maintained in flask by addition of ¾ volume of medium in 150 ml flask as per the method of Bert and Priefer (2004). Flasks were sealed with rubber septum and incubated at 30^{o}C by shaking at 200 r.p.m. and the syringe was used to take out samples.

2.6: Estimation of Plant growth promoting factors

2.6.1: Culture conditions for EPS production and quantification

Fresh overnight grown culture of *Rhizobium* strains transformants as well as native obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30ºC was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g*

and washed thrice with sterile normal saline and used to for inoculation in 150ml flask containing 30ml M9 minimal medium supplemented with 50mM glucose as sole carbon source, cultures were grown at 30ºC for 24h used to examine the EPS production. EPS was extracted using (EDTA) method described by (Tapia et al., 2009). Bacterial cells were centrifuged at 14,000 rpm for 20min supernatant was used for EPS extraction. EDTA (2% v/v) was added to supernatant for precipitation and kept at 4^0C for 2h, EPS obtained in each extraction were separated by centrifugation from solution and under 4^0C. EPS containing free cells solution was obtained by filtrated using nitrocellulose of 0.22 mm of pore size (Millipore). Filtered solution was used for EPS estimation. The phenol-sulphuric method was followed for total EPS estimation EPS (expressed as µg/ml) (Dubois et al., 1956) reaction mixture contained 1ml of EPS solution,1ml of aqueous phenol and 5ml of concentrated H_2SO_4 was mixed properly and kept for 15-20minutes, absorbance was measured at 490nm, the amount of EPS was determine against glucose as standard.

2.6.2: Biofilm assay

Samples were inoculated in test tube containing 3ml M9 minimal medium containing 50mM glucose concentration, Bacterial cultures were incubated under aerobic conditions in Orbitek rotary shaker maintained at 30°C for 24h with agitation speed kept constant at 200rpm. Growth medium was discarded after 24h from each test tube; to remove the unattached bacteria with test tubes were washed with Phosphate Buffer Saline (PBS). Biofilm were fixed with 2 ml of 99% methanol, tubes were left empty to dry. Crystal violet 2 ml of 1% was used for staining of adhered cells with 5 minutes incubation. Surplus stain was rinsed by distilled water for 5 times. Tubes were air dried and the dye attached to cells was dissolved in 1.5 ml of 33% glacial acetic acid. Biofilm formation was detected by simple tube method and estimated by measuring optical density (OD) at 570nm spectrophotometer assay described by Mathur et al., (**2006**).

2.6.3: Indole acetic acid (IAA) production and estimation

Overnight grown all cultures were pulled in sterile 1.5ml eppendorff in laminar, aspetic conditions were maintained , samples were washed 3 times by normal saline, these samples were used for inoculation of IAA production, cultures were inoculated in 150ml conical flask containing 30ml M9 minimal medium with L-tryptophan (0.1%)

and one set without L-tryptophan with 50mM glucose as carbon source , flask were incubated at 30^0C orbital shaker 200rpm shaking for 24h, further samples were screened for IAA production, experiment was carried out in triplicates. Cell-free supernatant was obtained by centrifugation at 9000 × g for 20 minutes; supernatant was used for IAA extraction method described by (Sinha and Basu, 1981). To the 10 ml of supernatant, 2 ml of salkowski's reagent (1 ml of 0.5 M $FeCl_3$ + 50 ml of 35% perchloric acid) was added and incubated for 30 mintues under darkmess. Pink colour formed was determined colorimetrically at 540 nm as IAA production.

2.7: PQQ determination

PQQ production was estimated using the method of Rajpurohit et al., (2008). *Rhizobium* strains transformants and were grown for 36 h on M9 minimal medium containing 50 mM glucose. Cell supernatant was digested with 50% acetonitrile 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.

2.8: Analytical techniques

Modified Lowry's method (Peterson 1979) was used for total protein estimation. *Rhizobium* strains native as well as transformants increased in cell growth density were determined at OD600 (spectrophotometer). Increase in culture growth, pH drop indicates organic acid secretion and phosphate solubilisation from rock phosphate in medium, End point culture supernatants samples were filtered through 0.2 µm nylon membranes further they were used for Phosphate estimation by the ascorbate method (Ames **1966**) same samples were used for HPLC analysis to detect organic acid levels, retention time of acids were determined under above mentioned conditions. Standards organic acids were used for quantification (equipment from Shimadzu , India) HPLC was performed using a C18 column operated at room temperature with 0.01 M H_2SO_4 as mobile phase at a flow rate of 0.7 ml min^{-1}, while for citric acid estimation the same column was operated at room temperature using

mobile phase of 20mM Na_2HPO_4 with 2.5% acetonitrile at a flow rate of 1.0 ml min^{-1} and the column effluents were monitored using a UV detector at 210 nm (Buch et al., 2009, 2008) and measuring left over glucose using the GOD-POD kit (Enzopak, Reckon Diagnostics Pvt. Ltd., India). Physiological parameters were calculated like growth rate, biomass yield on glucose and specific glucose reduction rate as described by (Buch et al., 2009, 2008). Graph Pad Prism (version 3.0) and Microsoft Excel were used for statistical analysis of the parameters. Each parameter has been represented as mean ± SD or mean ± SEM as specified in the figure legends.

(i) Specific growth rate (h-1):

$$k = \frac{(\text{Log10Nt1} - \text{Log10Nt2}) \times 3.3}{(t1 - t2)\ (h)} \quad \text{where,}$$

N1 and N2 are the number of cells at time t1 and t2 respectively and (t1-t2) is the corresponding time interval in hours. 3.3 is the factor derived from the formula- number of generations (n) = (Log10N-Log10N0)/Log102. The number of cells was calculated from O.D.600nm using the correlation 1 O.D.600nm = 1.5x109 cell/ml (Koch et al., 2001)

(ii) Specific total glucose utilization rate (QGlc):

$$QGlc = \frac{\Delta\text{Glucose}\ (t1 - t2)\ (g/L)}{\Delta\text{dcw}\ (t1 - t2)\ (g/L) \times \text{Time interval}\ (t1 - t2)\ (h)} \quad \text{where,}$$

ΔGlucose (t1-t2) is the amount of glucose consumed over the time interval t1-t2; **Δ**dcw (t1-t2) is the difference in the dry cell weight (dcw) of the cells over the time interval t1-t2. QGlc is expressed as g glucose utilized/g dcw/h). Dry cell weight was calculated using the correlation 1 O.D.600nm = 0.382mg/ml (Bugg et al., 2000).

(iii) Biomass yield

$$Ydcw/Glc = \frac{\Delta dcw\ (t1\text{-}t2)\ (g/L)}{\Delta Glucose\ (t1\text{-}t2)\ (g/L) \times Time\ interval\ (t1\text{-}t2)\ (h)}$$ where,

All the parameters were as described for Specific glucose utilization rate. Ydcw/Glc is expressed as g of dry cell weight produced/ g glucose utilized/h.

(iv) Organic acid yield

$$Organic\ acid\ yield = \frac{Amount\ of\ organic\ acid\ produced\ (g/L)}{Total\ glucose\ utilized\ (g/L) \times dcw\ (g/L)}$$ where,

The amount of total glucose utilized was obtained by deducting the value of residual glucose concentration from the initial glucose concentration supplied in the medium. The difference between the total glucose utilized and gluconic acid produced was considered as glucose consumed. Hence, the total glucose utilized and not glucose consumed was taken into account for calculating specific glucose utilization rate. The statistical analysis of all the parameters was done using Graph Pad Prism (version 3.0) software and microsoft Excel.

Dry cell mass (dcw) = O.D600nm x 0.382, where 0.382 is the factor correlating O.D600 was with dry cell weight (Bugg et al., 2000)

2.9: Enzyme assays

2.9.1: Preparation of cells and cell free extracts

Glucose grown cells under above mentioned minimal media conditions were harvested in appropriate growth phase from 30ml of cell culture. The whole cell preparation for GDH assay was done by washing the harvested cells (mid-late log phase cultures) thrice with normal saline to remove the residual glucose of the medium and resuspending in 0.01M phosphate buffer (pH 6.0) with 5mM MgCl2.

2.9.2: Enzyme Assay Protocols

2.9.2.1: GDH (1.1.99.17) assay

GDH (D-glucose phenazine methosulphate oxidoreductase, (EC 1.1.5.2) was determined spectrophotometrically by following the coupled reduction of 2,6-dichlorophenolindophenol (DCIP) at 6_{00} nm (Quay et al., 1972). Molar absorbance of DCIP was taken as 15.1 mM^{-1} cm^{-1} at pH 8.75. The reaction mixture included: Tris-Cl buffer (pH 8.75), 16.66 mM; D-glucose, 66 mM; DCIP, sodium salt, 0.05 mM; phenazine methosulfate, 0.66 mM; sodium azide, 4 mM; whole cells, and distilled water to 3.0 ml.

2.9.2.2: Gluconate dehydrogenase assay (GADH) (1.1.99.3)

Rhizobium strains natives as well as transformants were grown on M9 and RP-containing minimal medium with 50 mM D-gluconate as the C source, Additionally RP-containing minimal medium contains 50mM HEPES buffer, pH 8.0, Cells were harvested (5000g for 10 min) after the pH dropped below 5.5, washed with sterile saline, and resuspended in 50 mM Tris-HCl, pH 8.75, and the whole-cell suspension was used as the source of enzyme in GADH assays. Spectrophotometrically method was followed for estimation of GADH activity according to Matsushita et al., (1982). Substrate of the GADH enzyme was done with D-gluconate, in the assay mixture. GADH activity was defined as nanomoles of 2, 6-dichlorophenolindophenol (DCIP) reduced per minute using D-gluconate, as substrates, respectively. Enzyme activity is defined as one unit of specific enzyme activity was defined as the amount of protein required to convert one nanomole of substrate per minute per milligram of total protein.

All the enzyme activities were determined at 30°C and were expressed per mg total protein. Total protein concentration of the crude extract as well as whole cell suspensions was measured by modified Lowry's method (Peterson, 1979) using bovine serum albumin as standard. Corrections were made for Tris buffer. Enzyme activities were calculated using following formula

$$\text{Specific enzyme activity (U)} = \frac{\Delta A_y \text{ nm/min}}{\epsilon \times \text{enzyme (sample) aliquot (ml)} \times \text{Total protein (mg/ml)}}$$

where,

ΔA_y nm is the difference in the absorbance at any given wavelengths (y nm) and ϵ is the millimolar extinction coefficient at *y* nm.

One unit of enzyme activity was defined as the amount of protein required to convert 1 nmole of substrate per minute unless stated in the figure legend.

2.10: Pot experiments - Interaction with (*Mung bean*).

2.10.1: Plant Inoculation Experiments:

Pure bacterial cultures were grown in nutrient broth at 30^{o}C, centrifuged, and diluted to a final concentration of 10^{8} CFU/ml in sterile distilled water. Mung bean seeds were surface sterilized in 70% ethanol for 1 min and in 1% sodium hypochlorite (NaClO) for 20 min, and then rinsed 5- 6 times with sterile distilled water and then allowed to germinate for 24 h on moist filter paper in petriplates kept in the dark at 30^{o}C.and then immersed in a bacterial suspension for 5 h. Germinated seeds were sown in pot with 10Kg of unsterilized locally collected soil. Before sowing seeds in pots initial physical and chemical properties in experimental of soil is presented in (**Table. 2.4).**The plants were grown at 30^{o}C in Green house and were irrigated with 50 mL of sterile distilled water every day. The experiment was conducted on six replicates (three pots per replicate, two plants per pot) for each treatment and was completely randomized. The plants were harvested after 45 days and growth parameters (plant height and plant biomass) were recorded. Total N, was measured by micro Kjeldahl's method, by Vepodest- Automatic Digestion, Distillation and titration system. Total P content was measured by Vanadomolybdo phosphoric acid yellow color method, and Total K content was measured by Flame photometer.

Leaf area

Leaf area of 3rd leaf was calculated according to method described by Khalilzadeh et al., 2012.

2.10.2. Acetylene Reduction Assay (ARA): Measuring Nitrogenase Activity

ARA assay was performed on GC at SICART method described by Castle 2010. Nitrogenase assays were conducted by acetylene reduction, using 0.1-mL samples injected into a PerkinElmer Clarus 680 Gas Chromatograph (GC) with dual flame ionization detectors (FID) were used in these experiments. Elite-U PLOT column. Acetylene was generated from calcium carbide and using 10% (w/v)

2.10.3. Chlorophyll Content:

Total cholophyll content of fresh leaves were estimated by using method described by (Khaleghi et al., 2012)

Table 2.4: Physical and chemical properties of experimental soil used for *Rhizobium* strains integrants

pH	P Kg/hac^{-1}	N Kg/hac^{-1}	K Kg/hac^{-1}
8	12.9	158.7	338.7

2.10.4.1: Antioxidant Enzymes / ROS scavenging enzyme activity:

(1) Superoxide Dismutase (SOD)

Two hundred mg of leaves were homogenized in a pre-chilled mortar and pestle under ice cold condition using 3.0 ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA and 1% (W/V) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay (Costa et al., 2002).

Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically based on inhibition in the photochemical reduction of nitroblue tetrazoilum (NBT). The 3 ml

reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0.1 ml enzyme extract, riboflavin was added last (Van Rossun et al., 1997).After addition of all these components and mixing, test tubes were placed on stand 30 cm below a light source consisting of four 15-w fluorescent lamps. The photochemical inhibition was allowed to happen for 10 minutes and stopped by switching off the light source. The photoreduction in NBT was measured as increase in absorbance at 560 nm. Blanks and controls were run the same way but without illumination and enzyme, respectively. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% in a reaction mixture. Enzyme unit of SOD was calculated according to formula given by Giannopolitis and Ries (1977).

$$\text{SOD unit} = \frac{\text{Control O.D. (without enzyme)}}{\text{Sample O.D.}} - 1 \times \frac{1}{\text{(g) conc. Enzyme}}$$

$$\text{SOD U/g protein} = \frac{\text{SOD unit}}{\text{protein mg/g}}$$

(2) Catalase

Two hundred mg of acetone powder homogenized with a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.2) with the addition of 1 mM EDTA and 1% (w/v) polyvinylpyrolidone (PVP) and a pinch of activated charcoal. The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants were used for the assay (Mahatma et al., 2011). Total catalase (EC 1.11.1.6) activity was determined in the supernatants by measuring the decrease in absorption at 240 nm as H_2O_2 (ε = 39.4 mM^{-1} cm^{-1}) got consumed according to the method of Aebi (1984) and enzyme activity expressed as mmol H_2O_2 oxidized min^{-1} g^{-1}protein. The 3 ml assay mixture contained 50 mM sodium phosphate buffer (pH 7.0), 30mM H_2O_2 and 50 μl enzyme extract. Enzyme unit of CAT was defined as:

$$\text{Mmol/min/g protein} = \frac{\text{O.D. } \Delta}{\varepsilon \times \text{protein mg/g} \times \text{Enzyme conc. (g)}}$$

Where, ε = Extinction coefficient, Enzyme conc. (g) = Amount of enzyme in 3 ml reaction mixture.

Guaiacol Peroxidase (POX)

Two hundred mg of leaves were homogenized in a pre-chilled mortar and pestle under ice cold condition in 2.0 ml of extraction buffer, containing 0.1M sodium phosphate buffer (pH 7.2) with the addition of 1 mM β-mercaptoethanol and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay.

POX (EC 1.11.1.7) activity was determined in the supernatants of centrifuged homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (€=26.6$mM^{-1}cm^{-1}$) in a reaction mixture containing 50 mM sodium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.05 ml enzyme extract, and 10 mM H_2O_2 (Costa et al., 2002).

(4) Ascorbate peroxidase (APX)

Mung bean leaf samples were crushed with chilled acetone in pre-chilled mortar-pestle. To obtain fine acetone powder the homogenates were filtered and stored immediately at - 20o C. Hundred mg of acetone powder was then homogenized in a pre-chilled mortar and pestle under ice cold condition using 2.0 ml of extraction buffer, containing 0.1 M sodium phosphate buffer (pH 7.2), and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20 minutes and the supernatants used for the assay (Mahatma et al., 2011).

APX (EC 1.11.1.11) activity was measured immediately in fresh crude extracts and assayed by procedure described by Nakano and Asada (1981). Three ml of the reaction mixture contained 50 mM sodium phosphate buffer pH 7.0, 0.1 mM H_2O_2, 0.5 mM ascorbic acid, 0.1 mM EDTA and 0.1 ml enzyme extract. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the

absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). The enzyme unit of APX was defined similar to that of CAT activity as described.

2.10.4.2: Estimation of Water Soluble Protein Content

Protein concentration of each enzyme extract was estimated by method of Lowry et al., (1951).

(a) Reagents for Lowry's Method

(i) Solution A: 2% Na2CO3 in 0.1 N NaOH

(ii) Solution B: (a) 1% CuSO4.5H2O solution

(b) 2% sodium potassium tartarate solution

Working solution of

B : Prepared fresh before use by mixing equal volume of solution B (a) and B (b).

(iii) Solution C: Prepared fresh before use by mixing 50 ml of solution A and 1 ml of working solution of B.

(iv) Solution D: Folin & Ciocalteu reagent (1N).

(b) Procedure (Folin Lowry's Method)

Enzyme extracts (25 µl) were taken in test tube and volume was made up to 1 ml with millipore water. A tube with 1 ml of water served as blank. Five ml of solution C was mixed by vortexing and kept for 10 min. Then 0.5 ml of solution D (Folin & Ciocalteu reagent) was added and vortexed. The tubes were allowed to stand at room temperature for 30 min. Absorbance was read at 660 nm. A standard curve was prepared using bovine serum albumin (BSA) in the concentration range of 10-80 µg.

Chapter 3

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

3.1: Introduction:

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

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

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

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

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

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

3.1.1: Rational of study

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

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

3.2 EXPERIMENTAL DESIGN

The experimental plan of work includes the following-

3.2.1: Bacterial strains used in this study

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

Table 3.1: Bacterial strains used in this study

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

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

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

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

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

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

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

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

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

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

3.3: Results:

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

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

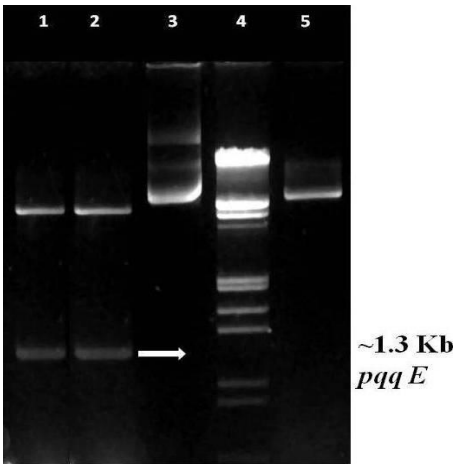


Fig. 3.1: Restriction endonuclease digestion pattern for *Rhizobium* transformants containing pJNK1: Lane 1 and 2 *Bj* pJNK1 and *Ml* pJNK1 BamH1

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

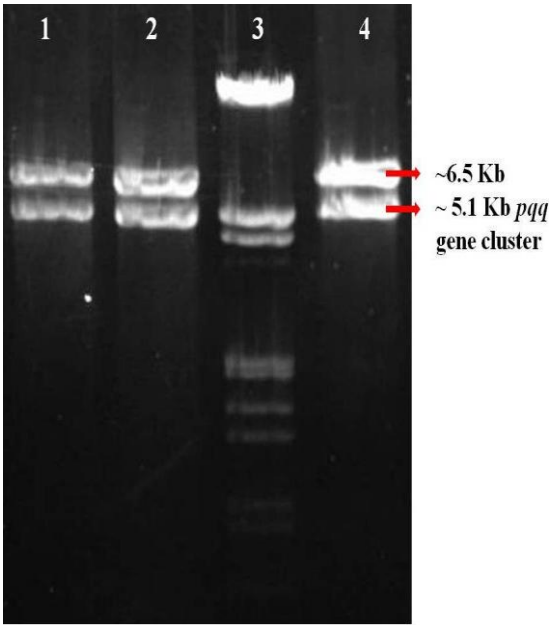


Fig. 3.2: Restriction endonuclease digestion pattern for *Rhizobium* transformants containing pJNK5 (Lane 1, 2 and 4 *Bj* pJNK5, *Ml* pJNK5 and *Sf* pJNK5 EcoR1- BamH1digested (6.5 kb and 5.1 kb), Lane 3- EcoR1/Hind III Marker)

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

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

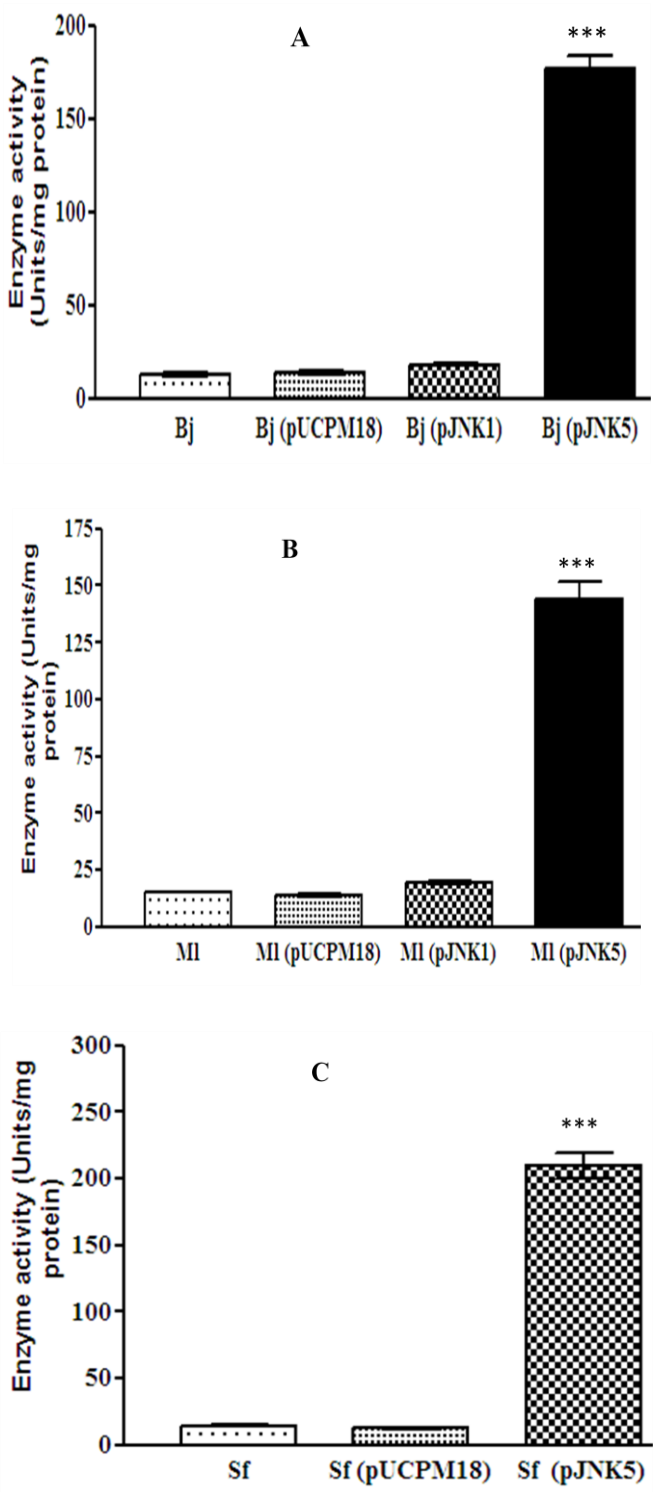


Fig 3.3: GDH activity of *Rhizobium* transformants containing *pqq* gene cluster (A) *B. japonicum*, (B) *M. loti* (C)*S. fredii* NGR 234. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

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

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

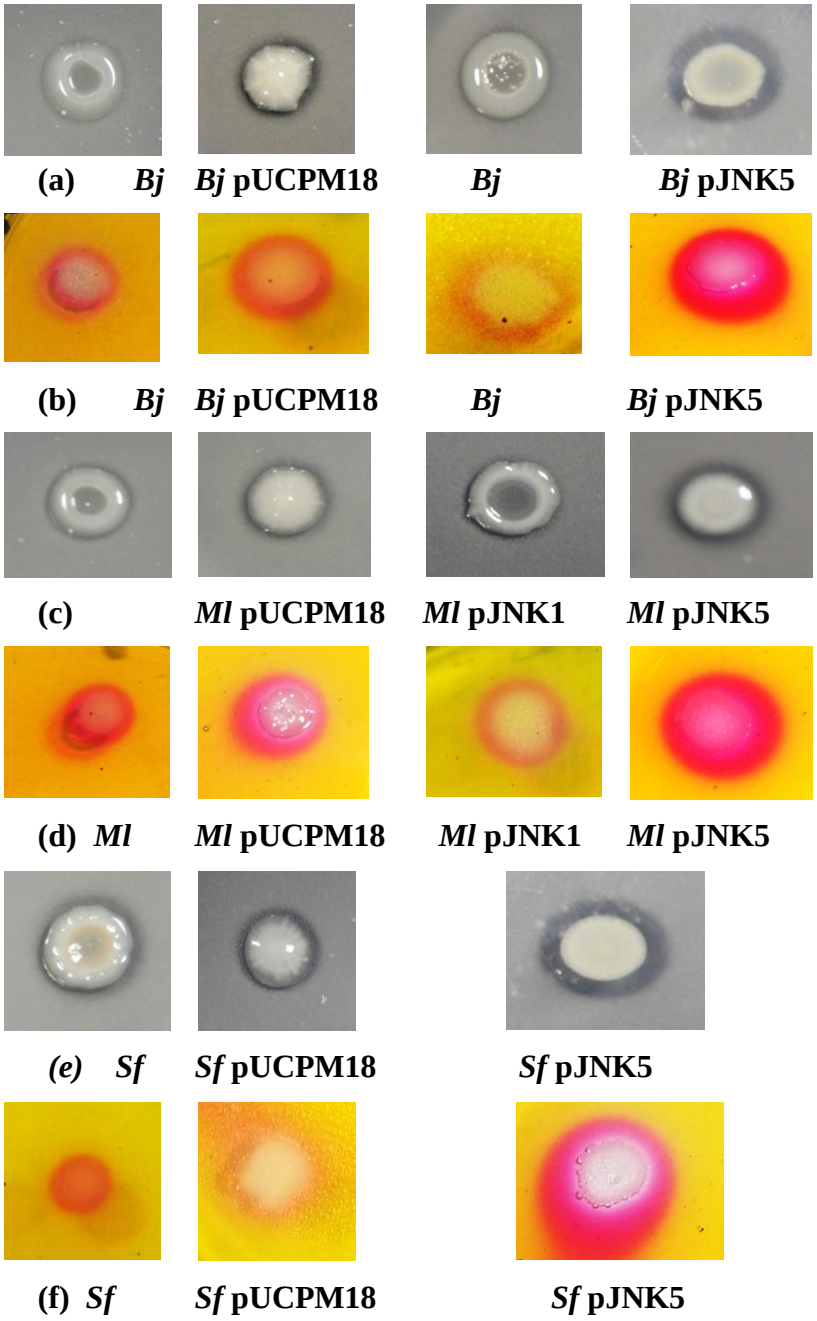


Fig 3.4: MPS phenotype of *B. japonicum*, *M. loti* and *S.fredii* strains harboring pJNK5 plasmid. (a), (c) and (e) on Pikovskaya's agar and **(b), (d) and (f)** Tris rock phosphate agar containing 50 mM glucose and 100 mM Tris HCl buffer pH 8.0. The results were noted after an incubation of 3 days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

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

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

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

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

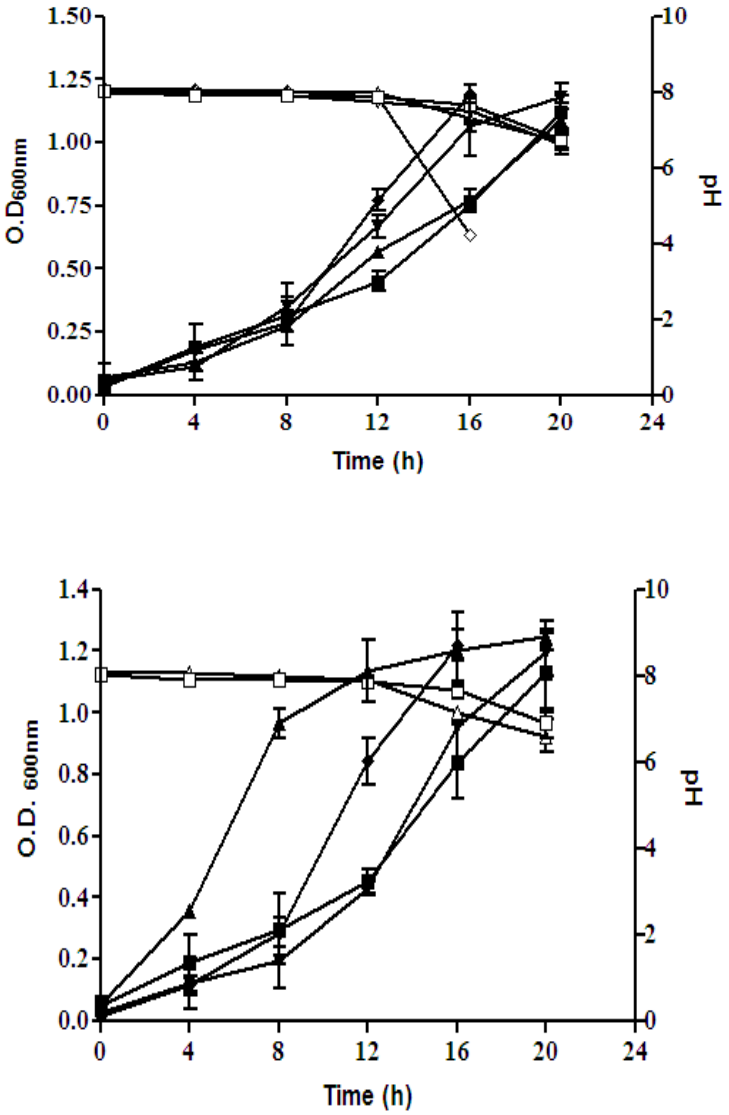


Fig. 3.5: Effect of *pqq* gene cluster overexpression on extracellular pH (□, Δ, ▽,◊) and growth profile (■, ▲, ▼,◆) of (A) *B. japonicum* and (B) *M. loti*, on TRP medium with 50 mM glucose .(□, ■, *Bj*, *Ml* wild type); {Δ, ▲, *Bj* (pUCPM18Gm^r), *Ml* (pUCPM18Gm^r)}; {▽, ▼, *Bj* (pJNK1), *Ml* (pJNK1)};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

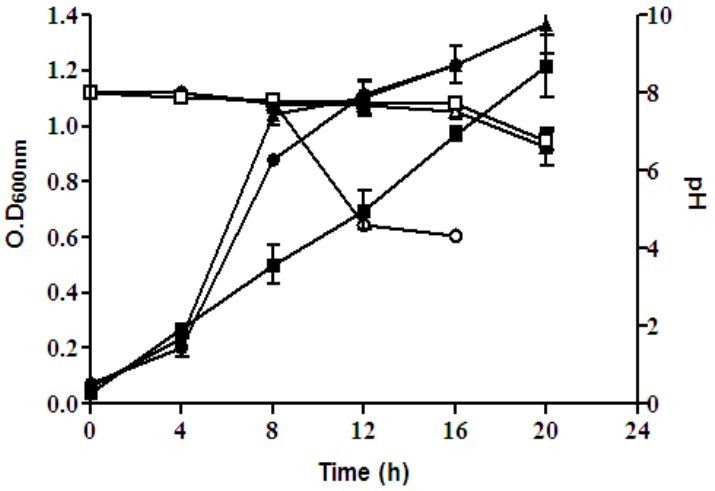


Fig 3.6: Effect of *pqq* gene cluster overexpression on extracellular pH (□, Δ, ○) and growth profile (■, ▲,●) of *S. fredii* on TRP medium with 50 mM glucose .(□, ■, *Sf* wild type); {Δ, ▲, *Sf* (pUCPM18Gm^{r}), {○, ●, *Sf* (pJNK5). OD_{600} and pH values at each time point are represented as the mean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

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

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

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

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

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

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

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

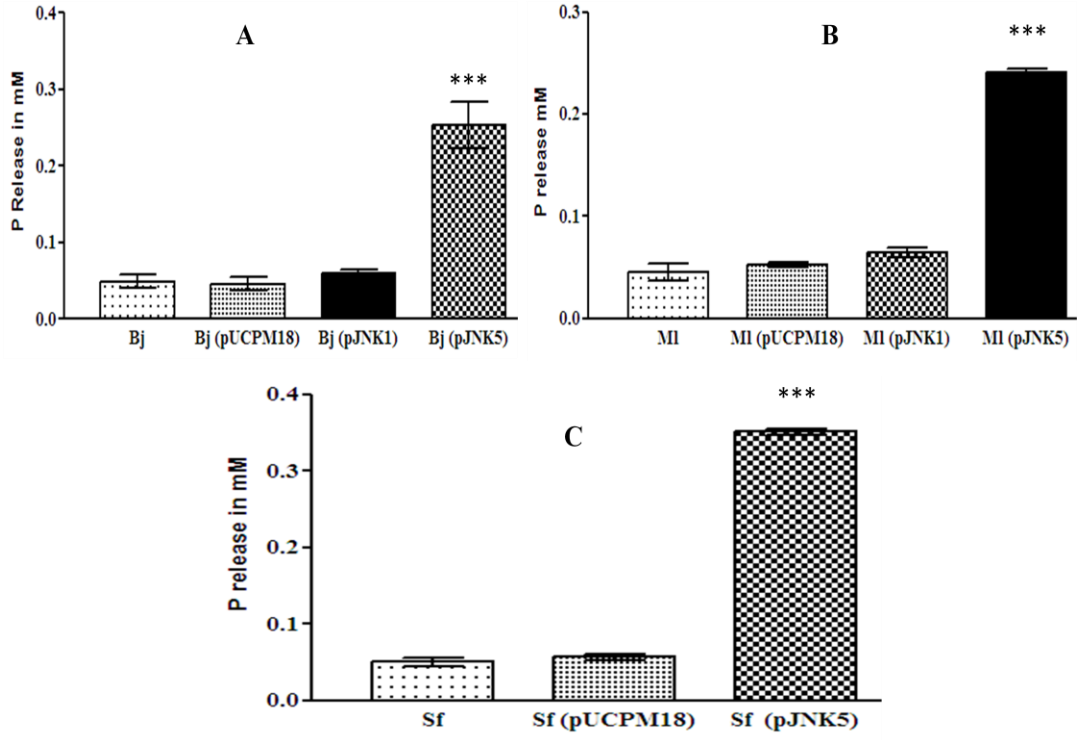


Fig.3.7: Phosphate released by *Rhizobium* transformants containing *pqq* gene cluster (A) *B. japonicum* (B) *M. loti* and (C) *S. fredii*. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

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

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

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

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

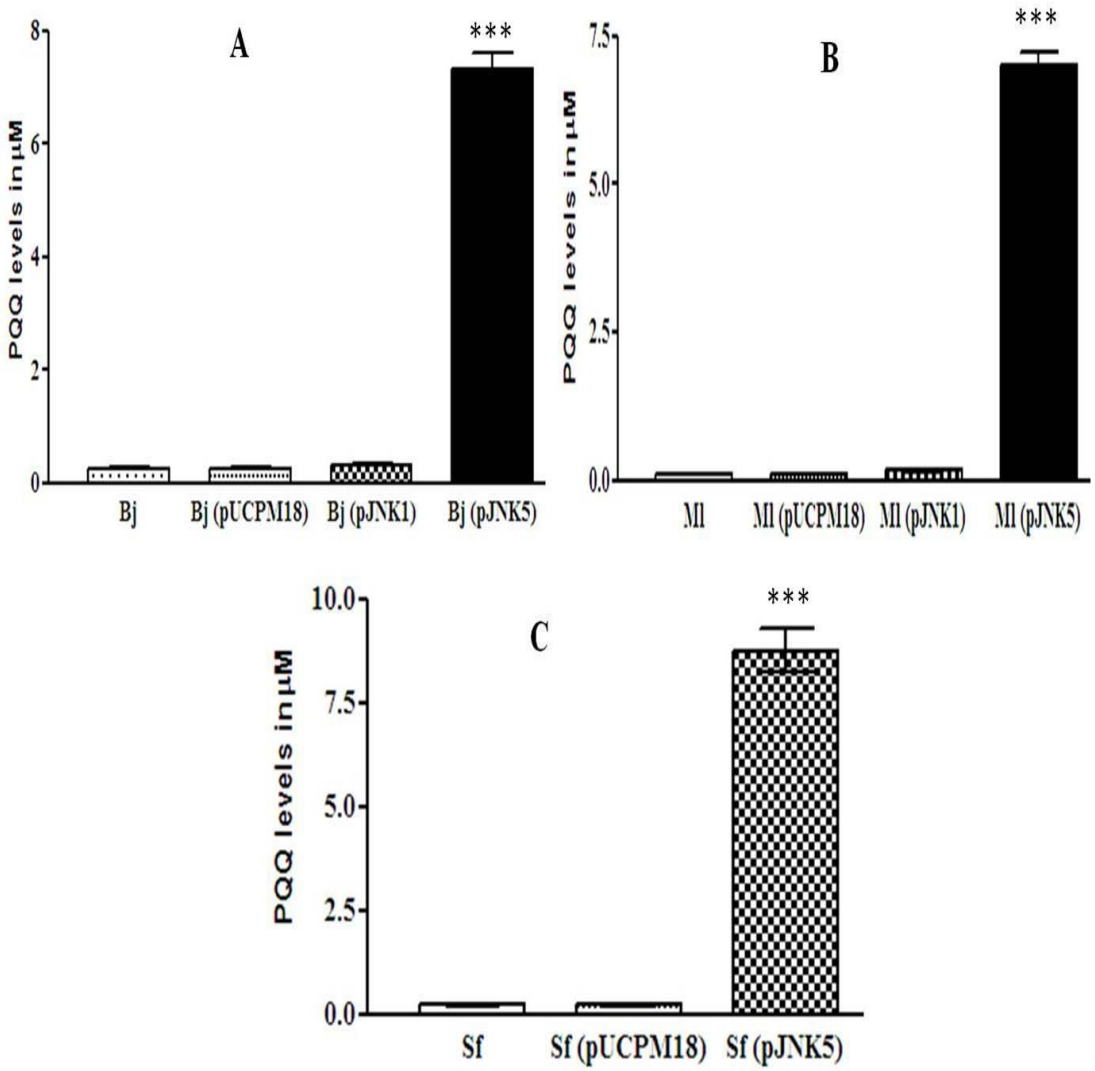


Fig. 3.8: PQQ secreted by *Rhizobium* transformants containing *pqq* gene cluster (A) *B. japonicum* (B) *M. loti* and (C) *S. fredii*. The results are expressed as Mean ± S.E.M of six independent observations * P<0.05, ** P<0.01 and *** P<0.001.

Table 3.5: Variation of PQQ secretion in bacteria.

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

Overexpression of *A. calcoaceticus pqq* gene cluster in *B. japonicum*, *M. loti* and *S. fredii* NGR234 enhanced EPS production. Phosphate plays as a positive regulator of EPS production, EPS production increased when supplemented with 0.1-20 mM P (Janczarek et al., 2011). *S. meliloti* produces two types of EPS, and the concentration of phosphate in the medium regulates the production of one type of EPS at the expense of the other. Under low-phosphate conditions EPS II predominates, and the colonies of these bacteria have a more mucoid morphology. Under normal conditions, *S. meliloti* produces EPS I, with less mucoid colonies (Zhan et al., 1991; Mendrygal & Gonzalez, 2000). EPS has been shown to play an essential role for the effective establishment of the symbiosis between *Rhizobium* sp. NGR234 and *L. leucocephala* or *M. atropurpureum* (Bomfeti et al., 2011). EPS mutant strains did not

show nodule formation with *Glycyrrhiza uralensis* (Wang et al., 2008). The strains with high levels of EPS production tend to be more tolerant to acidic conditions and salinity than strains that produce low EPS levels (Cunningham and Munns, 1984; Eaglesham et al., 1987; Xavier et al., 1998; Freitas et al., 2007; Xavier et al., 2007). Thus ~2.5 fold increase in EPS production will help in enhancing the movement of rhizobium infection thread and symbiosome formation. EPS along with LPS and BacA increase the competitiveness of *Rhizobium* nodulation. PGPR function of *Rhizobium* transformants.

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

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

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

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

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

Chapter 4

Determining the MPS ability of *Rhizobium* strains containing *A. calcoaceticus pqq* cluster and *P. putida gad* operon

4.1. Introduction

Genetic modification of apoGDH containing *Bradyrhizobium japonicum, Mesorhizobium loti* and *Sinorhizobium fredii* NGR234 with *Acinetobacter calcoaceticus pqq* gene cluster resulted in secretion of ~ 28 mM GA and released P 0.25 mM when grown on 50mM Glucose RP buffered medium. For phosphate solubilization, 2-KGA with pKa 2.6 is known to be much better as compared to GA with pKa 3.86. In addition to acidification, 2KGA can also chelate calcium in soils (Moghimi and Tate, 1978; Moghimi et al., 1978). GA is oxidized to 2-KGA by Gluconate dehydrogenase (GADH) in the periplasm (Yum et al., 1997; Toyama et al., 2007; Saichana et al., 2008). 2GA is secreted by many bacteria and fungi (Webley and Duff 1965; Yum et al., 1997; Walker et al., 2003; Saichana et al., 2008; Vyas and Gulati 2009; Gulati et al., 2010; Park et al., 2010). GADH was isolated and characterized from different species like *Pseudomonas, K. pneumoniae, S. marcescens,* and *E. cypripedii* ATCC 29267 (Matsushita and Ameyama, 1982; Yum et al., 1997; Toyama et al., 2007; Saichana et al., 2008). Three subunit containing FAD-dependent gluconate dehydrogenase (GADH) enzyme, is encoded by *gad* operon comprising of three genes namely FAD dependent gluconate dehydrogenase, cytochrome c and smallest third subunit of unknown function. Overexpression of *E. cypripedii* ATCC 29267 *gad* operon in *E. coli* led to secretion of high amount of 2-KGA (Yum et al., 1997). Tomato and wheat seedlings inoculated with *G. diazotrophicus* PAL5 showed GADH and GDH activity, showed intense acidification and phosphate solubilization (Crespo et al., 2011). Phosphate solubilizing new species *Enterobacter oryziphilus* sp. nov. and *Enterobacter oryzendophyticus* sp. nov. from rice endosphere secretes 2-KGA (Hardoim, et al., 2013). Enhanced P solubilization phenotype due to increased secretion of 2-KGA was observed in *E. asburiae* PSI3 when overexpressed with *Pseudomonas putida* KT 2440 *gad* operon (Kumar et al., 2013). Similarly, genetically modified *H. seropedicae* Z67 with *P. putida gad* operon and *A. calcoaceticus pqq* genes showed efficient P and K solubilisation and increase in Fresh weight, Dry weight N, P, K content upon inoculation to rice plants (Wagh, 2013).

Most of the *Rhizobium* species lack *gadh* operon thereby do not secrete 2-KGA. Thus, the present study investigates the effect of heterologus overexpression of

A. calcoaceticus pqq gene cluster along with *Pseudomonas putida gad* operon in *B. japonicum, M. loti* and *S. fredii* NGR234 for 2-KGA and its effect on MPS ability.

4.2: EXPERIMENTAL DESIGN

The experimental plan of work includes the following-

4.2.1: Bacterial strains used in this study

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

Table 4.1: Bacterial strains used in this study

Plasmid/Bacterial strains	**Characteristics**	**Source/Reference**
***E. coli* strains**		
E. coli DH10B	Used to maintain plasmids for routine use	Invitrogen, USA
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r (100µg/ml)	Hester et al., 2000
pJNK6	pJNK5, Gm^r (20µg/ml) with *gad* operon 3.8 Kb of *P. putida* KT 2440	Wagh, 2013
***Rhizobium* strains**		
Bradyrhizobium japonicum USDA110	Accession number NC_004463.1	NCBI
Mesorhizobium loti MAFF030669	Accession number NC_002678.2	NCBI
Sinorhizobium fredii NGR234	Accession number NC_012587.1	NCBI
***Bj* pUCPM18**	*B. japonicum* with pUCPM18, Gm^r (control vector)	This study
***Bj* pJNK6**	*B. japonicum* with pJNK6Gm^r (20µg/ml)	This study
***Ml* pUCPM18**	*M. loti* with pUCPM18, Gm^r (control vector)	This study
***Ml* pJNK6**	*M. loti* with pJNK6Gm^r (20µg/ml)	This study
***Sf* pUCPM18**	*S. fredii* with pUCPM18, Gm^r (control vector)	This study
***Sf* pJNK6**	*S. fredii* with pJNK6Gm^r (20µg/ml)	This study

Table 4.1: List of bacterial strains used. Detailed characteristics of these strains are given in **Table 2.1** Parent strains and the transformants of *E. coli* and *Rhizobium* were respectively grown at 37ºC and 30ºC with variations in gentamycin and erythromycin concentrations for rich and minimal media as described in **Section 2.2.**

4.2.2: Development of *B. japonicum, M. loti* and *S. fredii* strains harboring *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon (pJNK6).

The recombinant plasmids pUCPM18 Gm (control) and pJNK6 were transformed in *B. japonicum, M. loti* and *S. fredii* by electroporation (Section 2.4.2.2). The transformants were selected on gentamycin selection plates and were confirmed by Restriction endonuclease digestion pattern.

4.2.3: Growth and MPS phenotype of pJNK6 transformant strains of *Rhizobium*

The MPS ability of *B. japonicum, M. loti* and *S. fredii* and its transformants were monitored on Pikovaskya's (PVK) agar and 100 mM Tris buffered RP (TRP) agar as described in **chapter 2**

4.2.4: Effect of heterologous *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon (pJNK6) overexpression on the physiology and glucose metabolism.

B. japonicum, M. loti and *S. fredii* transformants were subjected to physiological experiments involving growth and organic acid production profiles on TRP medium with 50 mM glucose as carbon source.The samples withdrawn at regular interval were analyzed for O.D.600nm, pH, extracellular glucose and organic acid as mentioned in chapter2. The physiological parameters were calculated as mentioned in chapter 2. The enzyme assays were performed as described chapter 2.

4.3: Results

4.3.1: Heterologous overexpression of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon (pJNK6) in *Rhizobium* strains.

The pJNK6 plasmid was incorporated *in B. japonicum, M. loti and S. fredii* NGR 234 strains and the transformants were confirmed based on restriction endonuclease digestion pattern (**Fig. 4.1).**

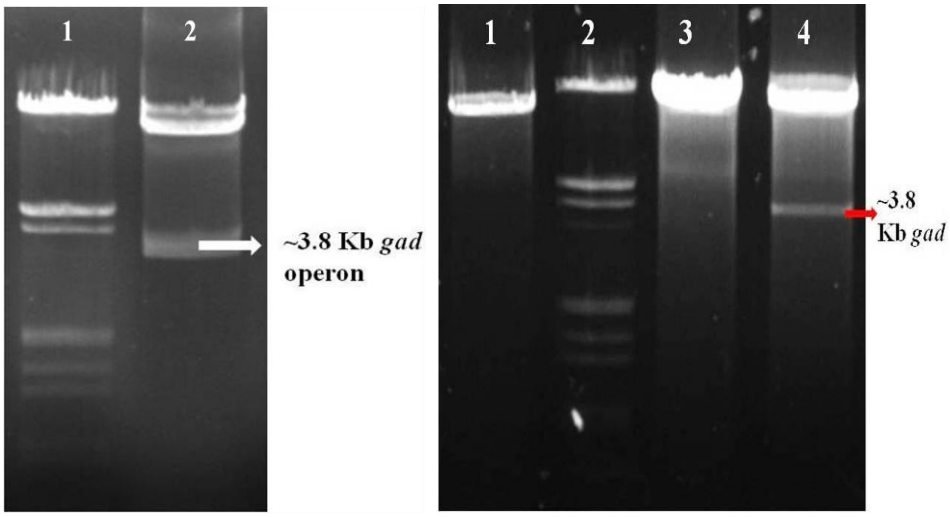


Fig. 4.1: Restriction endonuclease digestion pattern for *Rhizobium* transformants containing pJNK6 (Lane 1- EcoR1/Hind III Marker, Lane- 2, 4 and 6 *Bj* pJNK6, *Ml* pJNK6 and *Sf* pJNK6 BamH1/ XbaI digested (12.1 kb and 3.8 kb).

4.3.2 Effect of heterologous overexpression of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon (pJNK6) in *Rhizobium* strains on GADH and GDH activity in *B. japonicum, M. loti and S. fredii* NGR 234.

In *Bj* (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) GADH activity was found to be 340.9 U, 438.4 U and 326.3 U, respectively, which is 42, 73 and 36 fold increase compared to native strain. In transformants, GDH activity was found to be 182.8 U, 151.2 U and 219.0 U, respectively, with a fold increase of 14.51,10.10 and 15.24 compared to native strains, which is similar to *Rhizobium* strains transformed with pJNK5 alone

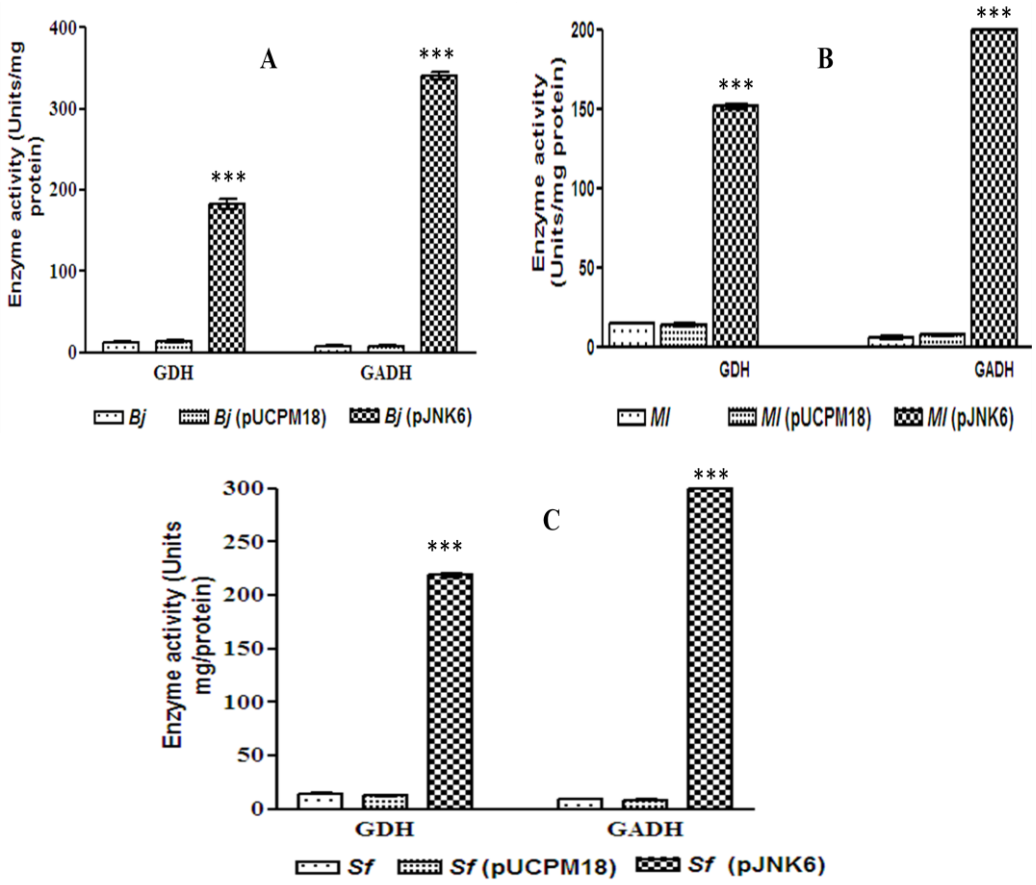


Fig 4.2: GDH and GADH activity of *Rhizobium* transformants containing *pqq* gene cluster (A) *B. japonicum*, (B) *M. loti* (C) *S. fredii* NGR 234. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

4.3.3: Growth and MPS ability of *Rhizobium* transformants of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon (pJNK6)

Bj (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) transformants showed good phenotype compared to native and control both on PVK and TRP plates after 3 days of incubation at 30°C. The pJNK6 transformants of *B. japonicum*, *M. loti* and *S. fredii* showed maximum zone of clearance as compared to the control pUCPM18 and pJNK5 transformants (**Table 4.2).**

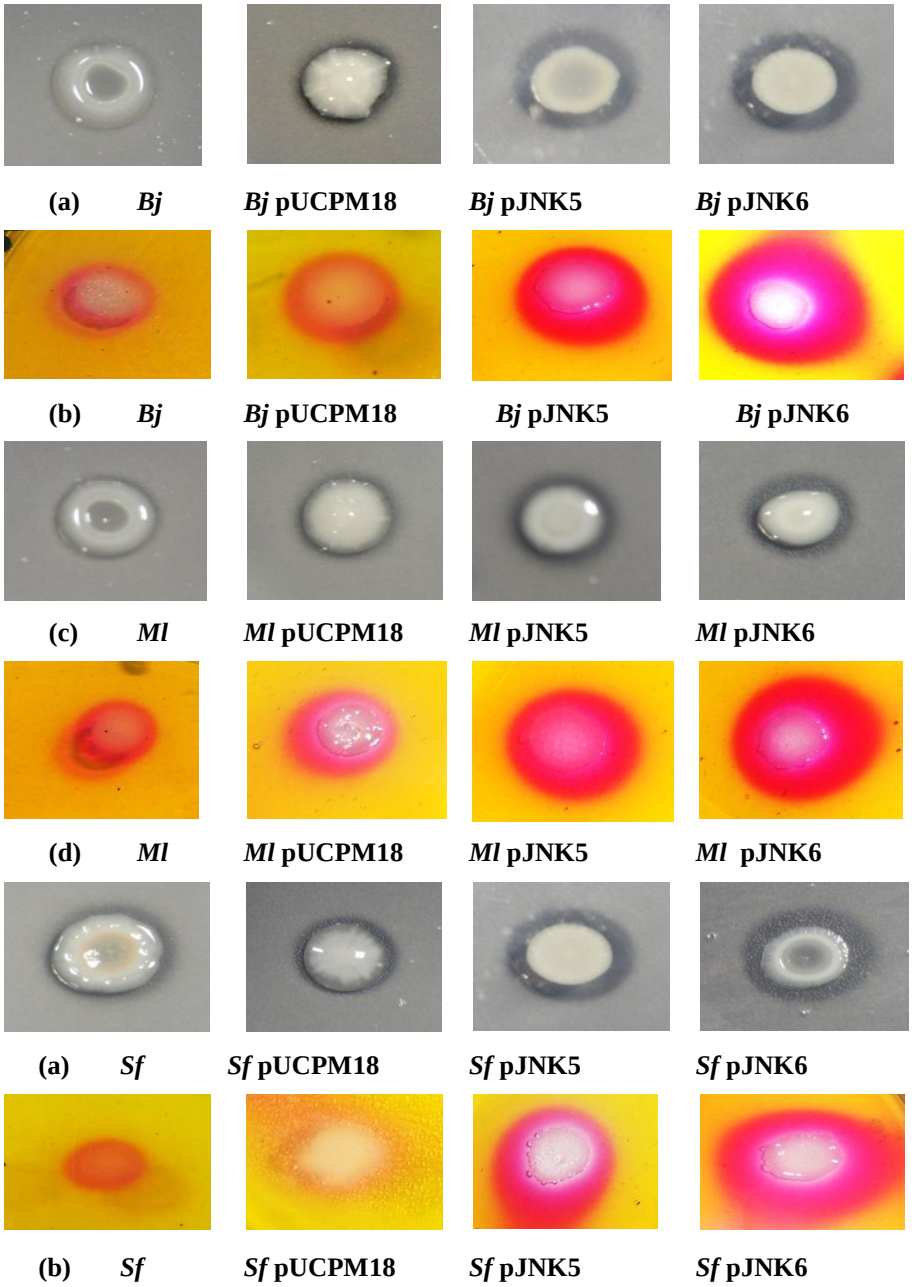


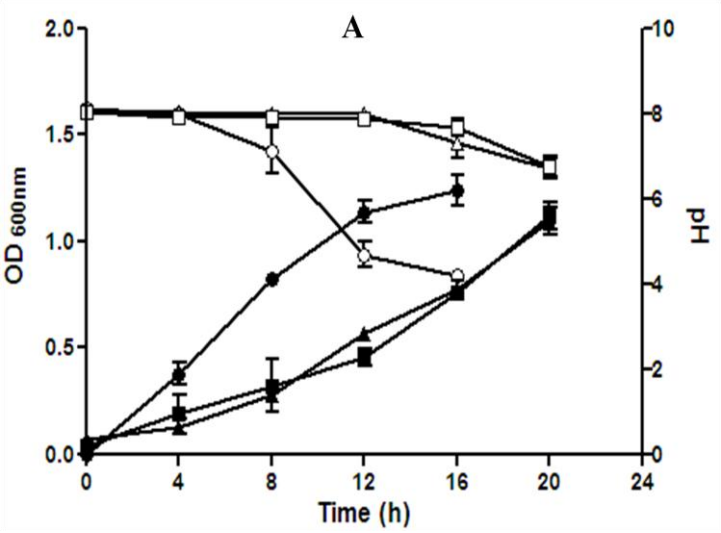
Fig. 4.3: MPS phenotype of *B. japonicum, M. loti* and *S. fredii* strains harboring pJNK6 plasmid. (a), (c) and (e) on Pikovskaya's agar and **(b), (d) and (f)** Tris rock phosphate agar containing 50 mM glucose and 100 mM Tris HCl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 ºC. Media composition and other experimental details are as described chapter 2.

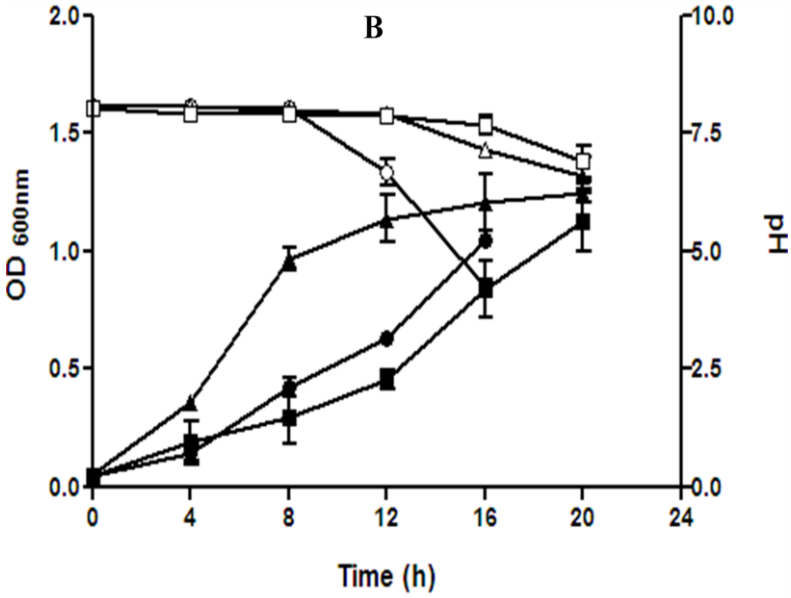
Rhizobium Strains	**Diameter of zone of clearance (mm)**	**Diameter of colony (mm)**	**Phosphate Solubilizing Index**
Bj	12.17 ± 0.29	11.17 ± 0.29	1.09
***Bj* pUCPM18**	11.17 ± 0.29	9.50 ± 0.50	1.22
***Bj* pJNK5**	14.50 ± 0.50	10.17 ± 0.29	1.44
***Bj* pJNK6**	14.17± 0.29	9.50 ± 0.50	1.55
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
***Ml* pUCPM18**	12.17 ± 0.29	10.17 ± 0.29	1.22
***Ml* pJNK5**	12.50 ± 0.50	9.17 ± 0.29	1.36
***Ml* pJNK6**	12.83 ± 0.29	8.83 ± 0.29	1.45
Sf	12.17 ± 0.29	10.50 ± 0.50	1.20
***Sf* pUCPM18**	12.17 ± 0.29	10.17 ± 0.29	1.22
***Sf* pJNK5**	14.50 ± 0.50	11.17 ± 0.29	1.29
***Sf* pJNK6**	14.50 ± 0.50	10.17 ± 0.29	1.42

Table 4.2: P solubilization index on Pikovskyas agar of *B. japonicum, M. loti and S. fredii* transformants. *Bj, Ml and Sf*: wild type strain; *Bj* pUCPM18, *Ml* pUCPM18 and *Sf* pUCPM18: *B. japonicum, M. loti and S. fredii* with vector control and *Bj* (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) **:** *B. japonicum* with *pqq* and *gad* gene cluster, *M. loti* with *pqq* and *gad* gene cluster and *Sf* with *pqq* and *gad* gene cluster. The results were noted after an incubation of 3 days at 30 ºC and are given as mean ± S.D. of three independent observations as compared to native *Bj, Ml* and *Sf.* The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

4.3.4: Effect of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon (pJNK6) overexpression on growth pattern pH profile and physiological effects in presence of 50mM glucose concentration.

pJNK6 transformants of *B. japonicum, M. loti* and *S. fredii* showed maximum growth and pH drop of 4.21, 4.21 and 4.23 respectively within 16 h compared to around pH 6.5 to 6.7 of native and control at 20 h **(Fig 4.4).**


A
OD 600nm
pH
Time (h)
2.0
1.5
1.0
0.5
0.0
10
8
6
4
2
0
0
4
8
12
16
20
24


B
OD 600nm
pH
Time (h)
2.0
1.5
1.0
0.5
0.0
10.0
7.5
5.0
2.5
0.0
0
4
8
12
16
20
24

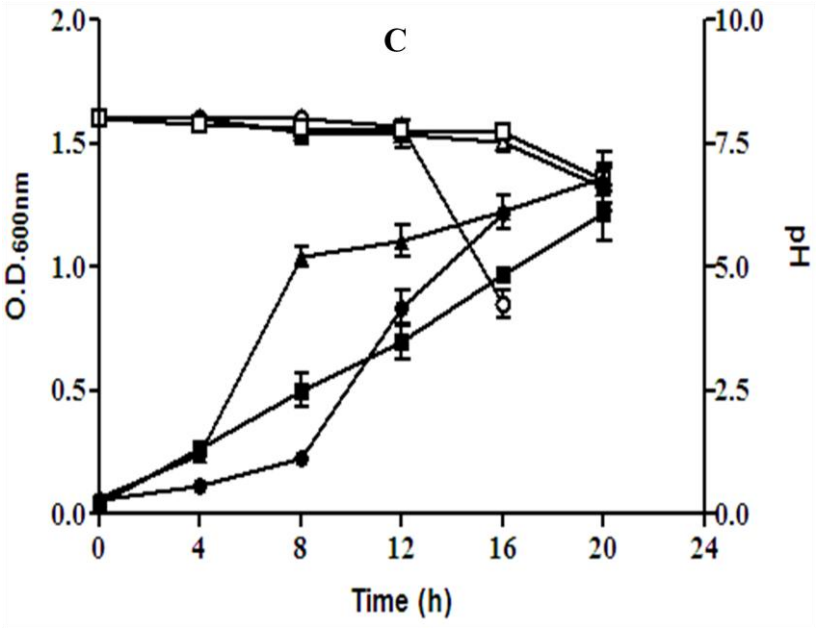


Fig. 4.4 : Effect of *pqq* gene cluster and *gad* operon overexpression on extracellular pH (□, Δ, ○) and growth profile (■, ▲,●) of (A) *B. japonicum* and (B) *M. loti*, (C) *S. fredii* on TRP medium with 50 mM glucose .(□, ■, *Bj*, *Ml*, *Sf* wild type); {Δ, ▲, *Bj* (pUCPM18Gm^r), *Ml* (pUCPM18Gm^r), *Sf* (pUCPM18Gm^r)}; {○, ●, *Bj* (pJNK6), *Ml* (pJNK6), *Sf* (pJNK6)};. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

In presence of 50 mM glucose, increase in GDH and GADH activity significantly affected growth profile. The total glucose utilization rate at the time of pH drop remained unaffected but there was ~ 2 fold decrease in glucose consumed due to increase in GA and 2-KGA acid secretion. However, the Specific Glucose Utilization Rate Q_{Glc} (g.g $dcw^{-1}.h^{-1}$) decreased by ~1.2 to ~1.9 fold in pJNK6 transformants. Additionally, the increase in GDH and GADH activity increased the specific growth rate by ~1.8 to ~2.0 fold, and improved the biomass yield by ~1.3 to ~ 1.6 fold in pJNK6 transformants compared to control (**Table 4.3**).

Rhizobium Strains	**Specific Growth Rate K(h^{-1}) [a]**	**Total Glucose Utilized (mM) [b]**	**Glucose Consumed (mM) [b]**	**Biomass Yield $Y_{dcw/Glc}$ (g/g) [a]**	**Specific Glucose Utilization Rate Q_{Glc} (g.g $dcw^{-1}.h^{-1}$) [a]**
Bj	0.186 ± 0.026	46.20 ± 0.20	38.23 ±1.33	1.78 ± 0.14	0.14 ± 0.01
Bj pUCPM18	0.280 ± 0.051	48.30 ± 0.46	40.65 ± 0.30	1.21 ± 0.16	0.21 ± 0.02
Bj pJNK6	0.363 ± 0.07***	49.10 ± 0.26	14.20 ± 1.57	2.39 ± 0.48**	0.11 ± 0.02
Ml	0.221 ± 0.03	45.91 ± 0.64	37.07 ± 0.55	1.36 ±0.26	0.19 ± 0.04
Ml pUCPM18	0.265 ± 0.02	48.40 ± 0.36	40.38 ± 0.02	1.0 ±0.04	0.26 ± 0.01
Ml pJNK6	0.438 ± .05***	49.67 ± 0.12	15.89 ± 0.22	1.96 ± 0.18*	0.13 ± 0.01
Sf	0.260 ± 0.02	46.10 ± 0.42	37.17 ± 0.55	1.69 ± 0.22	0.15 ± 0.02
Sf pUCPM18	0.311 ± 0.02	48.10 ± 0.10	40.67 ± 0.40	1.85 ± 0.10	0.14 ± 0.01
Sf pJNK6	0.468 ± .03***	49.03 ± 0.06	16.10 ± 0.21	2.68 ± 0.25**	0.09 ± 0.01

Table 4.3: Physiological variables and metabolic data from of *B. japonicum* , *M. loti* and *S. fredii pqq* transformants grown on TRP medium 100mM Tris-Cl buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml. The results are expressed as Mean ± S.E.M of six independent observations. [a] Biomass yield $Y_{dcw/Glc}$, specific growth rate (k) and specific glucose utilization rate (Q_{Glc}) were determined from mid log phase of each experiment. [b] Total glucose utilized and glucose consumed were determined at the time of pH drop. The difference between total glucose utilized and glucose consumed is as explained. * P<0.05, ** P<0.01 and *** P<0.001.

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

Significant increase was seen in P release by pJNK6 transformants of *B. japonicum, M. loti* and *S. fredii* compared to control and native, 13, 12 and 12 fold, respectively, in TRP medium in presence of 50mM glucose and 100mM Tris Cl Buffer pH 8.0 (**Fig. 4.5**).

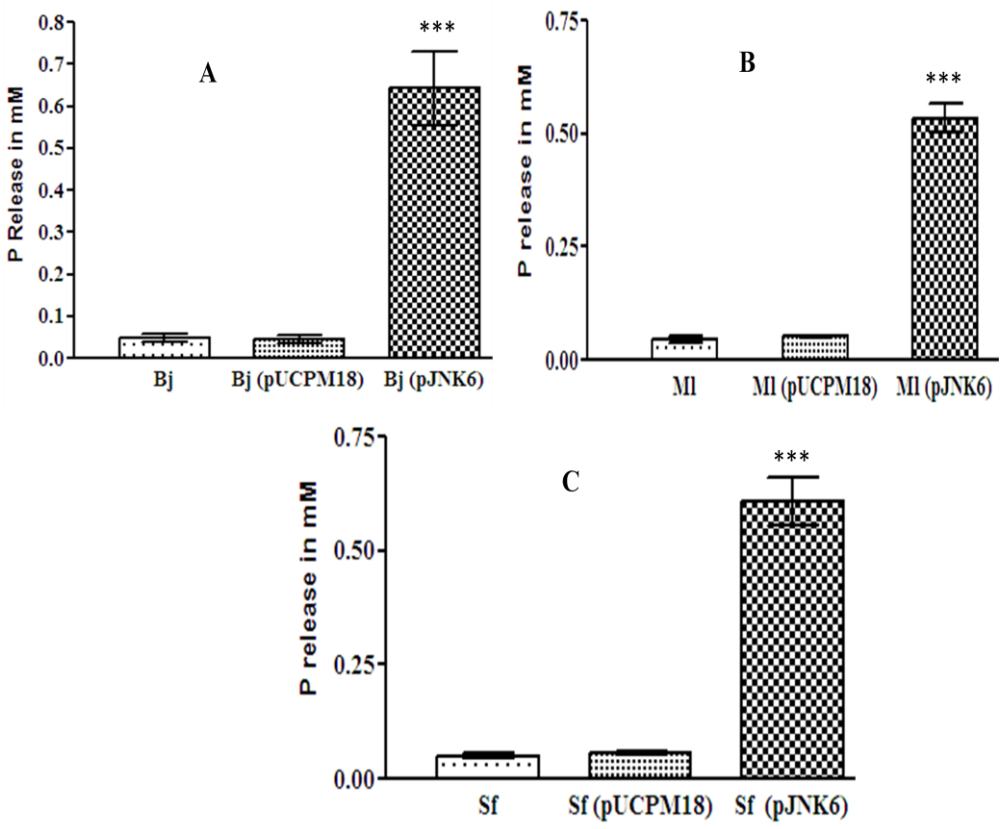


Fig.4.5: Phosphate released by *Rhizobium* transformants containing *pqq* gene cluster and *gad* operon (A) B. *japonicum* (B) *M. loti* and (C) *S. fredii*. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

Extracellular medium of pJNK6 transformants of *B. japonicum*, *M. loti* and *S. fredii* had 6, 5.27 and 6.83 folds higher amounts of 2-ketogluconic acid, respectively, with its specific yield ($Y_{C/G}$) increased by 4.06, 3.07 and 2.74 fold whereas levels of gluconic acid were 3.31, 3.07 and 2.64 folds higher with specific yield ($Y_{C/G}$) increased by 2.02, 1.82 and 1.13 fold compared to native and controls (**Table 4.4**)

Rhizobium Strain	**GA mM**	**2-KGA mM**	**Specific Yield GA $Y_{C/G}$**	**Specific Yield 2-KGA $Y_{C/G}$**
Bj	6.26 ± 0.23	2.37 ± 0.47	1.39 ± 0.12	0.49 ± 0.09
Bj pUCPM18	5.72 ± 0.06	1.93 ± 0.07	1.39 ± 0.08	0.46 ± 0.05
Bj pJNK6	20.73± .82***	14.17 ± 0.23***	3.11 ±0.15***	1.99 ±0.12***
Ml	6.53 ± 0.18	2.60 ± 0.20	1.01 ± 0.21	0.40 ± 0.1
Ml pUCPM18	5.80 ± 0.10	2.21 ± 0.13	0.98 ± 0.03	0.36 ± 0.04
Ml pJNK6	20.06± .24***	13.72 ± .22***	1.84 ± 0.03**	1.23 ±0.04***
Sf	6.760 ± 0.10	2.20 ± 0.15	1.59 ± 0.05	0.54 ± 0.10
Sf pUCPM18	5.50 ± 0.30	1.92 ± 0.03	1.10 ± 0.11	0.37 ± 0.01
Sf pJNK6	17.90±0.17***	15.03 ± .05***	1.81 ± 0.12**	1.48 ±0.07***

Table 4.4: Organic acid Secretion and Organic acid yield from of *B. japonicum* , *M. loti* and *S. fredii pqq* transformants grown on TRP medium 100mM Tris-Cl buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

Bj (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) also secreted ~7.30 µM, ~7.00 µM and ~8.75 µM PQQ, which is similar to *Rhizobium* strains transformed with pJNK5 alone (**Fig. 4.6**).

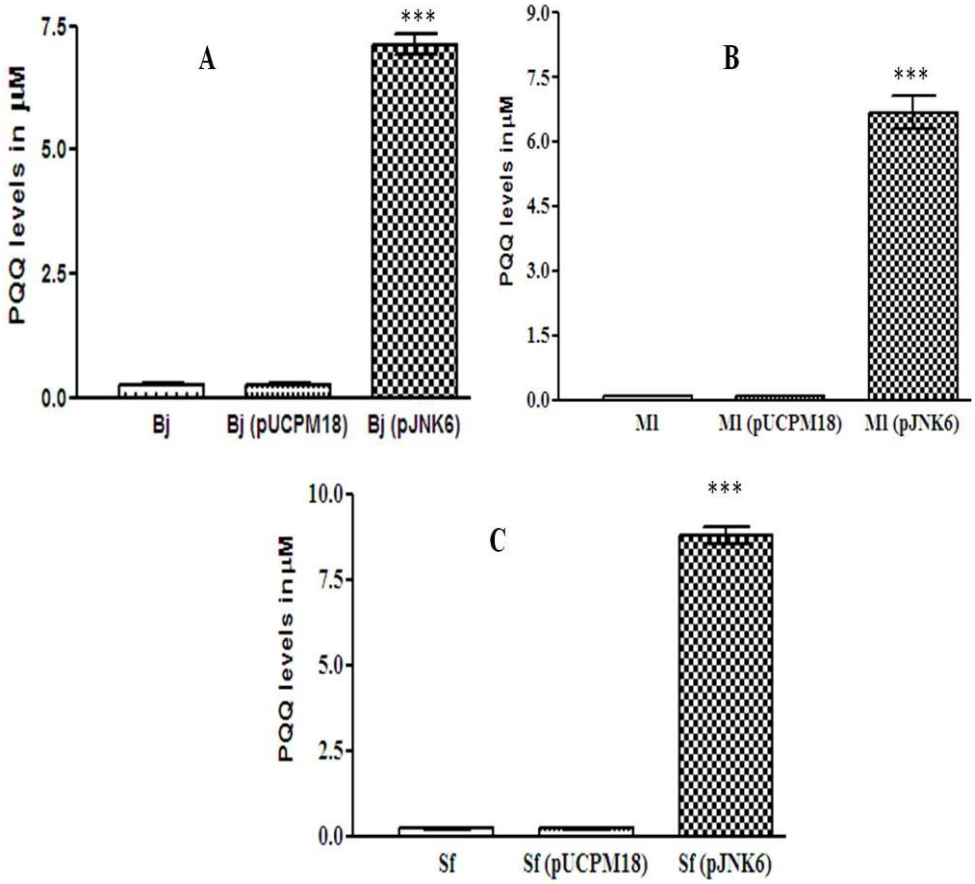


Fig. 4.6: PQQ secreted by *Rhizobium* transformants containing *pqq* gene cluster and *gad* operon (A) *B. japonicum* (B) *M. loti* and (C) *S. fredii*. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

4.3.8: Effect of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon (pJNK6) overexpression on Biofilm, EPS and IAA secretion in *Rhizobium* transformants.

Biofilm and EPS synthesis on an average showed significant increase by ~2.5 fold, while ~ 1.8 fold increase in IAA secretion in *Bj* (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) transformants in 100 mM Tris-Cl buffer pH 8.0, 50 mM glucose and rock phosphate 1 mg/ml in comparison to native and control (**Table 4.5**).

***Rhizobium* Strains**	**Biofilm O.D.at 550nm**	**EPS (g/100ml)**	**IAA (µg/ml)**
Bj	1.39 ± 0.02	11.48 ± 0.1	20.14 ± 1.33
***Bj* pUCPM18**	1.45 ± 0.03	12.56 ± 0.2	22.32 ± 1.65
***Bj* pJNK6**	4.67 ± 0.03***	28.45 ± 0.2***	42.69 ± 1.34***
Ml	1.51 ± 0.06	11.34 ± 0.05	28.17 ± 1.35
***Ml* pUCPM18**	1.81 ± 0.05	11.89 ± 0.1	29.67 ± 1.81
***Ml* pJNK6**	5.64 ± 0.04***	33.33 ± 0.5***	47.22 ± 2.12***
Sf	1.61 ± 0.10	12.53 ± 1.54	32.79 ± 1.87
***Sf* pUCPM18**	2.10 ± 0.16	13.43 ± 1.11	34.10 ± 1.32
***Sf* pJNK6**	6.12 ± 0.2***	38.11 ± 1.88***	52.12 ± 1.22***

Table 4.5: Biofilm, EPS and IAA production by *Bj* (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) transformants in TRP medium. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

Discussion

Rhizobium species lack GADH enzyme which converts GA to 2-KGA. Transformation of *B. japonicum, M. loti* and *S. fredii* NGR 234 strains with pUCPM18 Gm^r containing *A. calcoaceticus pqq* gene cluster under constitutive P*lac* promoter along with *P. putida* KT2440 *gad* operon with its own promoter showed GADH activity ~390 U, while GDH activity was ~190 U with 50 mM glucose in M9 medium. Incorporation of *E. cypripedii* ATCC 29267 *gad* operon in *E. coli* increased GADH activity from ~410 U to ~2150 U in presence of gluconate (Yum et al., 1997). Similar increase in GAD activity (1738 U) was found in *E. asburiae* PSI3 *gad* transformant in presence of gluconate (Kumar et al., 2013). GADH activity of *H. seropedicae* Z67 when overexpressed with *A. calcoaceticus pqq* gene cluster along with *P. putida* KT2440 *gad* operon (*Hs* pJNK6) was ~414 U, in HRP minimal medium which is similar to *E. asburiae* PSI3 *gad* transformant (438 U) in TRP medium (Wagh, 2013). *Bj* (pJNK6), *Ml* (pJNK6) and *Sf* (pJNK6) showed similar expression of *P. putida* KT2440 *gad* operon as with *E. asburiae* PSI3 and *Hs* (pJNK6), this shows that *P. putida* KT2440 *gad* operon is functional in *Rhizobium* strains.

Hs pJNK6 secreted 15.83 mM 2-KGA along with 3.79 mM GA, when grown on 100 mM HEPES containing 50 mM Glucose (Wagh, 2013). Overexpression of *P. putida* KT2440 *gad* operon alone in *E. asburiae* PSI3 possessing high level of PQQ biosynthesis secreted 11.63 mM 2-KGA along with 21.65 mM GA (Kumar et al., 2013). *E. cypripedii* ATCC 29267 *gad* operon in *E. coli* could secrete ~ 13mM 2KGA in LB containing 100mM glucose (Yum et al., 1997).Our study shows similar results as seen in different species, *Rhizobium* strains secreted up to 15 mM 2-KGA along ~21 mM GA, respectively, when grown on 50 mM glucose on TRP medium. *Rhizobium* strain oxidizes glucose chiefly using ED pathway, overexpression of *pqq* cluster and *gad* operon channelized glucose towards direct oxidation pathway, resulting in to secreting higher amounts of GA and 2-KGA, this change in glucose flux in no way affected growth rate and biomass yield .

Hs pJNK6 released 0.457 mM P and ~1.1 μM PQQ (Wagh, 2013). *E. asburiae* PSI3 *gad* transformant could solubilize 0.84 mM rock phosphate in presence of 45 mM glucose while native stain requires 75mM glucose to solubilize rock phosphate in

TRP medium (Kumar et al., 2013). Our study showed similar results as *Hs* pJNK6, but P release was less compared to *E. asburiae* PSI3 *gad* transformant. The reason for this variation in P release in different species is not clear.

Increase in EPS, Biofilm and IAA secretion of *Rhizobium pqq-gad* transformants was similar to that of *pqq* transformants which could improve the nodulation, nitrogen fixation and plant growth promotion by *Rhizobium* strains.

Studies show that, genetic modification of *Rhizobium* species were predominantly done to increase nitrogen fixing ability or nodule formation (Vieira, et al., 2010). Using biotechnology tools, soil inhabiting indigenous species or Non PSMs can be converted to PSM. Most of the genetic modifications carried out till date is to enhance gluconic acid secretion, very few studies are there for 2-KGA secretion (**Table 4.6**) (Kumar et al., 2013). *Rhizobium* strains expressing *A. calcoaceticus pqq* cluster and *P. putida gad* operon togather for 2-KGA secretion would make *Rhizobium* strains very efficient P solubilzer. For stable genetic modification, genomic integration of *pqq-gad* operon is desirable in *Rhizobium* strains.

Table 4.6 Genetic modifications for enhanced MPS by bacteria

Gene/Function	Source	Host	Mineral P solubilized	Organic acid	References
PQQ biosynthesis	*S. marcescens*	*E. coli*	TCP	GA	Krishnaraj and Goldstein (2001)
pqqE	*E. herbicola*	*Azospirillum sp.*	TCP	GA ?	Vikram et al. (2007)
pqqED genes	*R. aquatilis*	*E. coli*	HAP	GA	Kim et al. (1998)
Unknown	*E. agglomerans*	*E. coli*		GA ?	Kim et al. (1997)
pqqABCDEF genes	*E. intermedium*	*E. coli* DH5α	HAP	GA	Kim et al. (2003)
Ppts-gcd, P gnlA-gcd	*E. coli*	*A. vinelandii*	TCP	GA	Sashidhara and Podille (2009)
gabY Putative PQQ transporter	*P. cepacia*	*E. coli* HB101		GA	Babu-Khan et al. (1995)
Unknown	*Erwinia herbicola*	*E. coli* HB101	TCP	GA	Goldstein and Liu (1987)
gltA/ citrate synthase	*E. coli* K12	*P. fluorescens* ATCC 13525	DCP	Citric acid	Buch et al (2009)
Unknown	*Synechocystis* PCC 6803	*E. coli* DH5α	RP	Unknown	Gyaneshwar et al 1998

NADH insensitive cs /Na^+ dependent citrate transporter operon	*E. coli* /*S.typhimurium*	Genomic integration in *P. fluorescens* PfO-1	RP	Citric acid	Adhikary, 2012
gad / Gluconate dehydrogenase	*P. putida* KT2440	*E. asburiae* PSI3	RP	GA and 2KGA	Kumar et al 2013
pqq/gad PQQ/ GADH	*A. calcoaceticus* /*P. putida* KT2440	*H. seropedicae* Z67	RP	GA and 2KGA	Wagh, 2013
pqq/gad PQQ/ GADH	*A. calcoaceticus* /*P. putida* KT2440	*B.japonicum. M. loti* and *S. fredii* NGR234	RP	GA and 2KGA	This Study

GA- Gluconic acid, 2KG- 2 ketogluconic acid, DCP- Dicalcium phosphate, TCP- Tricalcium phosphate, RP- Senegal rock phosphate, HAP- Hydroxyapatite.

Chapter 5

Genomic integration of *A. calcoaceticus pqq* cluster and *P. putida gad* operon with *vgb, egfp* in *B. japonicum* USDA110, *M. loti* MAFF303099 and *S. fredii* NGR 234

5.1 Introduction

Presence of plasmids in *Azotobacter vinelandii*, *Azospirillum brasilense*, and *Pseudomonas putida* GR12-2 showed impaired growth, phosphate solubilization, nitrogen fixation, siderophore production, and indole acetic acid biosynthesis, under various experimental conditions (Glick 1995). Diverse effects were found in physiology, cellular metabolism, alteration in ATP biosynthesis as well as perturbations in host DNA replication, transcription, and translation in *E. coli* due to presence of plasmid (Rozkov et al. 2004; Wang et al. 2006; Chou 2007; Ow et al. 2006; 2009). *P. fluorescens* WCS365 and *P. putida* GR12-2 containing plasmid showed reduced stability and competitiveness under rhizospheric conditions, respectively (Simons et al. 1996; Schmidt-Eisenlohr et al., 2003). Additionally, presence of antibiotic marker and plasmid instability impose problem to use genetically modified organism for field studies (Neubauer et al, 2003; De Gelder, 2007). Previous reports from our laboratory showed overexpression of genes in plasmids exerted greater metabolic alterations on *P. fluorescens* ATCC 13525 metabolism despite of low copy number (Buch et al., 2010). Similarly, presence of plasmids adversely affected growth and gluconic acid secretion of phosphate solubilizing *E. asburiae* PSI3 under phosphorus limited condition (Sharma et al., 2011). Thus to overcome the drawbacks of plasmid mediated genetic manipulation, incorporation of genes into the genome becomes an attractive strategy (Sibley and Raleigh, 2012).

Genomic integration by additional copies of *nif*A and *dct*ABD in *Rhizobium meliloti* increased, root colonization, nodule occupancy and alfalfa yield in the field condition (Bosworth et al., 1994). *B. cepacia* G4 TOM- (toluene *o*-monooxygenase) was stably integrated in chromosome of *Rhizobium* strains (ATCC 10320 and ATCC 35645), engineered bacteria degraded trichloroethylene and were competitive against the unengineered hosts in wheat and barley rhizospheres (Shim et al., 2000). *Sphinomonas* CAR-1.9-dioxygenase gene constitutively integrated in *R. tropici*, degraded 48 % dioxin within first 3 days in field condition (Saiki et al., 2003). Genetic manipulation of *R. leguminosarum* bv. *trifolii* containing multiple copies of *ros*R and *pss*A genes responsible for EPS production, resulted in increased growth rate, EPS production and number of nodules (Janczarek et al., 2009). ACC deaminase

gene integrated in to *M. loti* expressing ACC deaminase only in nodules, expressed the gene constitutively and showed more number of nodules and competitiveness compared to wild type on *L. japonicus* (Conforte et al., 2010).

Majority of genetic manipulations in *Rhizobium* species are done to enhance its nodulation competitiveness, nitrogen fixation ability, biocontrol activity , with stand biotic-abiotic stresses and rhizoremediation (Rengel, 2002; Archana et al., 2010; Mabrouk and Belhadj 2010; Singh et al., 2011). There are no reports of genetic manipulation of *Rhizobium* species for mineral phosphate solubilization. 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, 2.5 fold increase in P release compared to pJNK5 transformants was observed. Higher 2KGA secretion increased MPS efficiency of *Rhizobium* transformants. It would be interesting to study genomic integration of *A. calcoaceticus pqq* gene cluster , *Pseudomonas putida gad* operon , *Vitreoscilla hemoglobin* (*vgb*) and *egfp* using Tn7 based integration system at *att* site in *B. japonicum, M. loti* and *S. fredii* strains and characterize the biochemical, growth and MPS abilities of the integrants.

Single copy tagging of bacteria without, any deleterious effects at neutral and unique site is carried out by using mini-Tn7 transposon system. The Tn7 transposon was originally discovered by Barth et al. (1976) on the plasmid R483 (IncIα) as an element carrying the resistance genes trimethoprim (TmR) and streptomycin/ spectinomycin (SmR/ SpR), which could be transposed to other replicons. These genes are flanked by the ends of the transposon, named the left (Tn7L) and the right (Tn7R) end (Lichtenstein and Brenner, 1982; Rogers et al., 1986). Tn7 inserts into specific location named the attTn7 site with high efficiency and unique orientation in *E. coli***,** located downstream of the coding region of enzyme glucosamine synthetase - *glm*S gene and thereby does not disrupt the gene (Gringauz, et al., 1988; Peters et al., 2001). Glucosamine synthetase is required for cell wall synthesis and it is conserved among many bacteria and, therefore Tn7 is likely to have the same specific insertion site in many different bacteria (Volger et al., 1989). The transposon genes require Trans functioning *tnsABCD* for specific insertion into the attTn7 site. Thus, sequences

located in the 3‟end of the coding region of *glm*S are recognised by transposase proteins directing the actual insertion into the attTn7 site, down-stream of the *glmS* gene**.** However, if this site is unavailable the transposon can insert into other sites with low frequency.

Gene complementation, gene expression analysis, strain construction, and reporter gene-tagging of *Pseudomonas aeruginosa* and *Yersinia pestis*, particularly in biofilm and animal model studies were done using mini-Tn7 transposon. Tn7-based delivery system was used to integrate genes encoding for different fluorescent protein and luciferase protein along with promoter into the chromosomes of *Erwinia chrysanthemi, P. fluorescens, P. syringae, P. putida* and many gram negative bacteria by te (Koch et al., 2001, Lambersten et al., 2004; Hee et al.,2008). Thus mini Tn7 transposition is a powerful technique for the single-copy integration or excision of a gene of interest at chromosomal level to carry out protein functional studies, gene expression analysis, insertional random mutagenesis, or gene-tagging of bacteria in living organisms.

Function of *Vitreoscilla hemoglobin* (VHb) is to enable the organism to survive in oxygen-limited environments by acting as an oxygen storage-trap or to facilitate oxygen diffusion to terminal oxidases under hypoxic conditions (Wakabayashi et al., 1986). Several cloning experiments showed that the presence and expression of VHb can improve not only cell growth but also productivity of recombinant protein (Khosravi et al, 1990; Geckil et al, 2004; Chung et al., 2009). Cell-free extracts of *A. calcoaceticus* showed low levels of GDH activity when grown on acetate-glucose mixture in oxygen-limited condition, activity got restored with introduction of oxygen (Van Schei etal., 1988). Genetically modified *Rhizobium* with *vgb* overexpression has enhanced the growth of *Rhizobium* and also increased nitrogen content in bean plants (Ramirez et al., 1999). In soil and nodules, amount of oxygen plays an important role for growth, nitrogenase activity, colonization etc.

The present study describes the genomic integration of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon , *V. hemoglobin* (*vgb*) and *egfp* using Tn7 based integration system at *att* site in *B. japonicum, M. loti* and *S. fredii* strains and compares its effect to plasmid based expression on growth amd MPS ability.

5.2 Experimental design

5.2.1: Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
pGRG36	Intergration vector (Tn7)	Gregory et al., 2006
pUCPM18	pUC18 derived Broad-Host-Range vector; Ap^r	Hester et al., 2000
E. coli DH10B	*Str^r F^- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZ ΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-*	(Invitrogen) USA
pJNK6	pUCPM18Gm^r with *pqq* gene cluster of *A. calcoaceticus* and *gad* operon of *P. putida* KT2440 under *plac*; Ap^r, Gm^r	Wagh, 2013
pJIPgv	pGRG36 with *pqq* gene cluster of *Acinetobacter calcoaceticus* and *gad* operon of *P. putida* KT2440 under *plac*; Ap^r, *vgb* gene and *egf* gene Ap^r	This study
Bj intPgv	Genomic integrant of *B. japonicum* containing *lac-pqq, gad, vgb, egfp* Ap^r	This study
Ml intPgv	Genomic integrant of *M. loti* containing *lac-pqq, gad, vgb, egfp* Ap^r	This study
Sf intPgv	Genomic integrant of *S. fredii* containing *lac-pqq, gad, vgb, egfp* Ap^r	This study

Table 5.1: Bacterial strains used in this study. The details of the plasmids and the concentration of the antibiotics used are given in the **Table 21** and **2.2**.

5.2.2: Cloning of *pqq- gad* operon in integration vector.

Construction of artificial *pqq gad* operon containing constitutive *lac* promoter, of *A. calcoaceticus* and *gad* operon of *P. putida* KT2440, *vhb* gene and *gfp* was done as per the following strategy. XT-20 polymerase was used for PCR amplification (Bangalore Genei, India) from plasmid pJNK6. Sequence of forward (*lac*) rimerTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACAGGA

GGACGCATG GAATTCCACCATAAGTTTTGAC3` and reverse primer of *gad* operon 5' AGT CGC TTCGCTGAA ATGTAGTGG3' was used to amplify *pqq- gad* operon. Plasmid pGRG36-mini-Tn7-Amp-*egfp* was digested with *Sma*I, gel purified

and ligated with 9 kb amplicon containing artificial *pqq- gad* operon was ligated with pGRG36 containing *vgb* and *egfp* **(Fig. 5.1).** Clone was confirmed by restriction digestion and PCR amplification. Resultant construct of 17.9 kb, named as pJIPgv consisted of artificial *pqq- gad* operon.

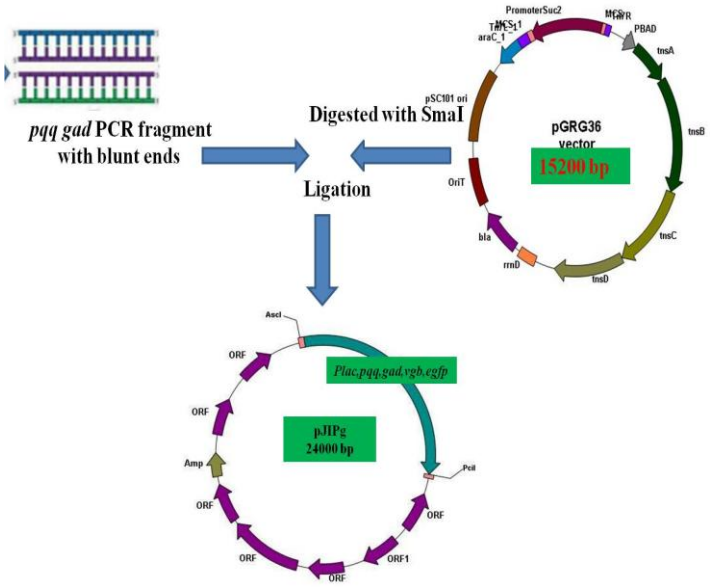


Fig. 5.1: Strategy used for cloning of *pqq-gad* gene cluster in pGRG36 containing *vhb, egfp* resulted in pJIPgv.

5.3: Results.

5.3.1: Construction of Genome integrants of *B. japonicum M. loti* and *S. fredii*

PCR amplification of *pqq* gene cluster along with *gad* operon under constitutive *lac* promoter was done from pJNK6 plasmid containing *pqq-gad* by using specific primers as mentioned above. This PCR amplicon was used for ligation with integration vector (**Fig. 5.2).** For further confirmation, the recombinant plasmid pJIPgv plasmid containing *pqq* gene cluster and *gad* operon under constitutive *lac* promoter was confirmed by restriction enzyme digestion. pJIPgv was digested with PvuII and pGRG36*vhb, egfp* plasmid digested with PvuII **(Fig. 5.3).**

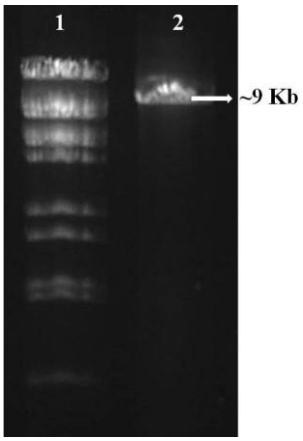


Fig. 5.2: PCR amplification of *pqq, gad* with constitutive *lac* promoter

Lane1- Marker **BstEII Digest**, Lane2- **PCR** amplification with constitutive *lac* promoter

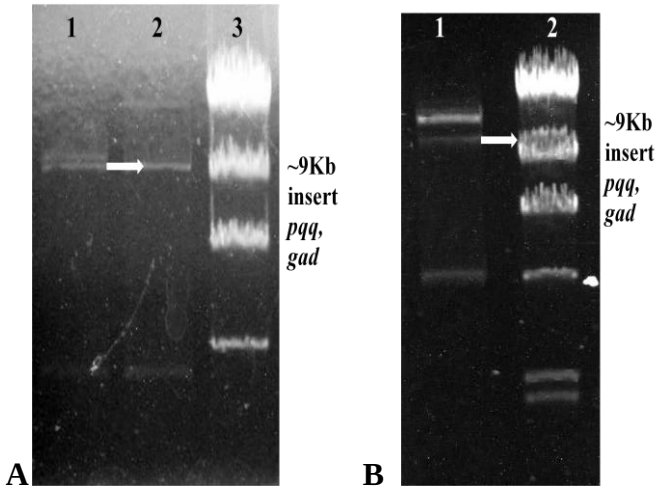


Fig.5.3: Restriction enzyme digestion pattern of pJIPgv plasmid containing *pqq* gene cluster and *gad* operon under *lac* promoter. (**A-B**) Lane1- pJIPgv digested with PvuII, Lane2- control plasmid pGRG36*vgb*, *egfp* digested with PvuII, Lane3 - λ DNA- Hind III Molecular weight marker.

B. japonicum, *M. loti* and *S. fredii* integrants were confirmed based on PCR amplification of *pqq-gad* from genomic DNA (**Fig. 5.4).**

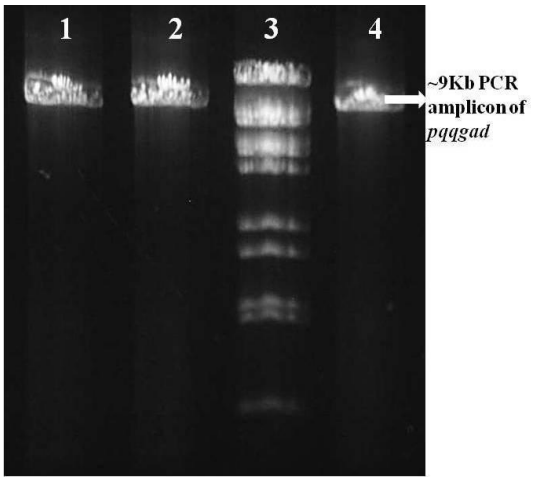


Fig. 5.4: Confirmation of Genome integrants by PCR amplification of *pqq* and *gad* from *B. japonicum, M. loti* and *S. fredii* integrants: Lane1,2 and 4 - PCR amplicon of *pqq,gad* from genomic DNA of *B. japonicum, M. loti* and *S. fredii* integrants.. Lane 3- λ DNA BstEII Molecular weight marker

5.3.2: GDH and GADH activity of *B. japonicum, M. loti* and *S. fredii* integrants.

In *B. japonicum M. loti* and *S. fredii* integrants GADH activity was found to be ~193.06 U, ~200.10 U and ~162.9 U, respectively, which is 24, 34 and 18 fold increase compared to native strain (**Fig.**). In integrants, GDH activity was found to be 129.83 U, 80.23 U and 147.03 U, respectively, with a fold increase of 10, 5 and 10 compared to native strains, respectively. GADH and GDH activity decreased by 1.5 - 2.0 fold compared to pJNK6 *Rhizobium* transformants.

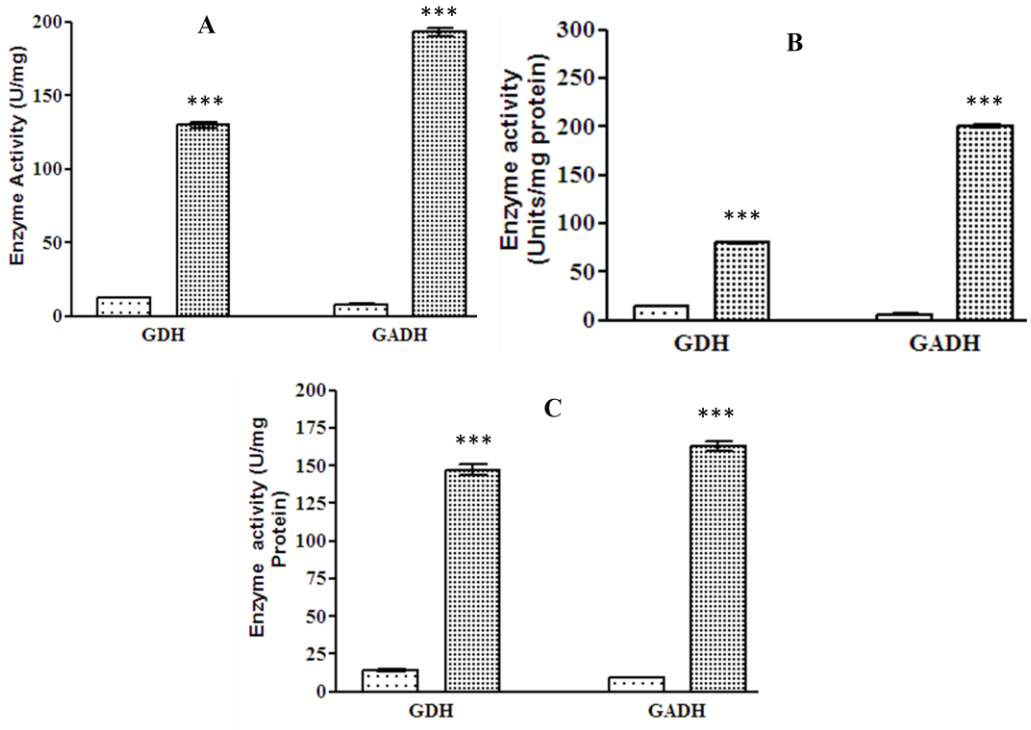


Fig. 5.5: GDH and GADH activity of *Rhizobium* integrants of *pqq* gene cluster and *gad* operon, *vgb* gene. (A) *Bj* intPgv **(B** *Ml* intPgv **(C)** *Sf* intPgv. Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

5.3.3: Growth and MPS ability of *B. japonicum, M. loti* and *S. fredii* integrant on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

B. japonicum M. loti and *S. fredii* integrants showed good phenotype compared to native and control both on PVK and TRP plates after 3 days of incubation at 30°C. Integrant showed compared to pJNK6 transformant decreased MPS phenotype on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate, while on PVK plates integrant showed stronger Ca-P solubilization **(Fig. ; Table 5.2).**

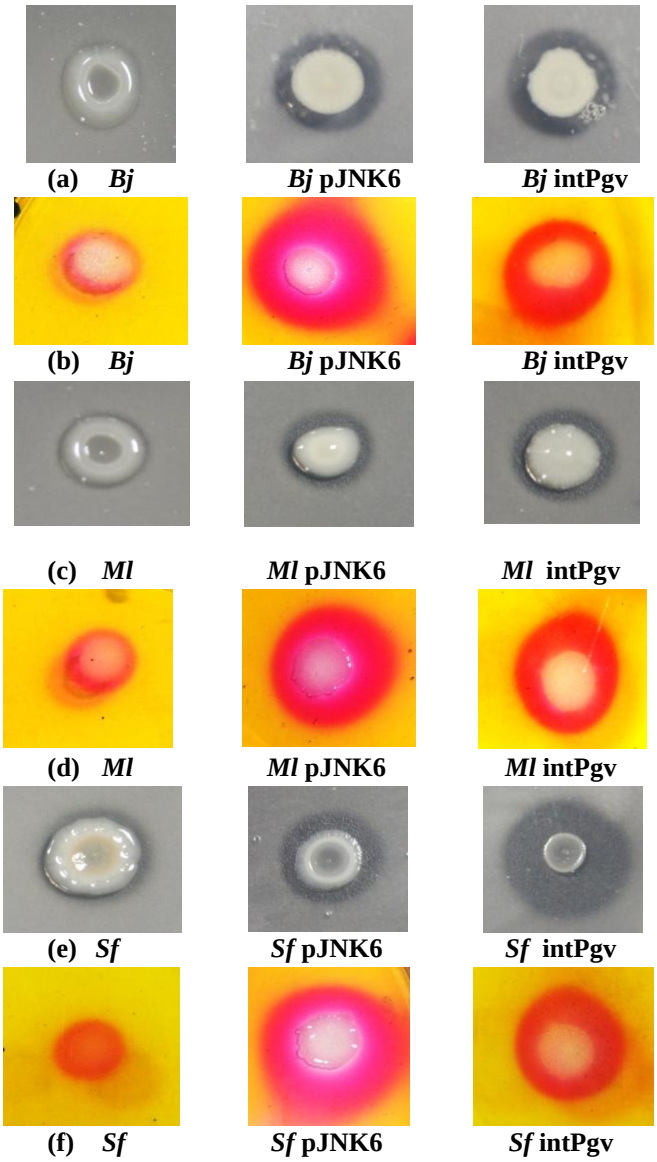


Fig. 5.6: MPS phenotype of *B. japonicum M. loti* and *S. fredii* integrants (a), (b) Zone of clearance formed by *Rhizobium* integrants on Pikovskaya's agar and (c), (d) zone of acidification Tris rock phosphate agar containing 50 mM glucose and 50 mM Tris Cl buffer pH 8.0. The results were noted after an incubation of 3 days at 30 ºC. Media composition and other experimental details are as described in Sections 2.2.4 and 2.7.

Rhizobium Strains	Diameter of zone of clearance (mm)	Diameter of colony (mm)	Phosphate Solubilizing Index
Bj	12.17 ± 0.29	11.17 ± 0.29	1.09
***Bj* pJNK6**	14.17± 0.29	9.50 ± 0.50	1.55
***Bj* intPgv**	14.50± 0.50	8.17 ± 0.29	1.77
Ml	12.83 ± 0.29	11.50 ± 0.50	1.09
***Ml* pJNK6**	12.83 ± 0.29	8.83 ± 0.29	1.45
***Ml* intPgv**	14.50 ± 0.50	9.17 ± 0.29	1.58
Sf	12.17 ± 0.29	10.50 ± 0.50	1.20
***Sf* pJNK6**	14.50 ± 0.50	10.17 ± 0.29	1.42
***Sf* intPgv**	20. 83 ± 0.29	6.50 ± 0.50	3.20

Table 5.2: P solubilization index on Pikovskyas agar of *B. japonicum, M. loti* and *S. fredii* integrants during 3 days of growth on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate. *Bj*, *Ml* and *Sf* : wild type strain; and *Bj* intPgv, *Ml* intPgv and *Sf* intPgv: *B. japonicum*, *M. loti* and *S. fredii* integrants. The results were noted after an incubation of 3 days at 30 ºC and are given as mean ± S.D. of three independent observations as compared to native *Bj*, *Ml* and *Sf*.

5.3.4: Growth pattern and pH profile of *B. japonicum, M. loti* and *S. fredii* integrants on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

The growth of *Bj* intPgv, *Ml* intPgv and *Sf* intPgv on 50 mM Tris-Cl buffer pH 8 and 50mM glucose reached to a maximum of 1.8 O.D. within 12 h in integrants compared to 20 h of the native *B. japonicum*, *M. loti* and *S. fredii* strains. pH of the medium dropped to 6.8, 6.8 and 5.7 within 20 h in the native *B. japonicum*, *M. loti* and *S. fredii* while pH drop to 4.1, 5.1 and 4.2 was seen within 12 h in *B. japonicum*, *M. loti* and *S. fredii* integrants, respectively (**Fig. 5.7 A,B,C**).

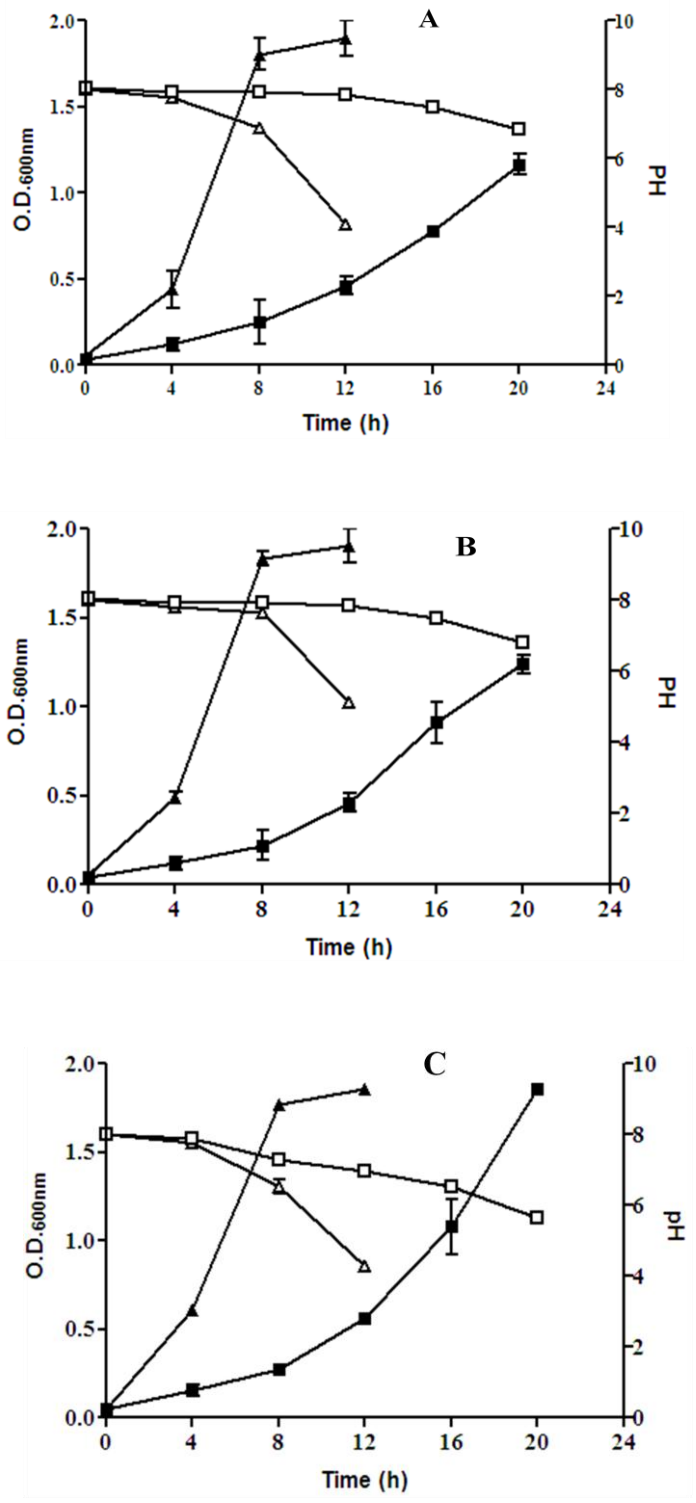


Fig. 5.7: Extracellular pH (□, ∆,) and growth profile on glucose 50 mM, Tris-Cl 50 mM rock phosphate medium (■, ▲,) of *B. jap*, *M. loti* and *S. fredii* integrants containing *pqq* gene cluster and *gad* operon, *vgb*, *egfp*. □, ■, *B. jap*, *M. loti* and *Sf* (wild type); ∆, ▲, *B. jap*, *M. loti* and *Sf* intPgv. OD_{600} and pH values at each time point are represented as the mean ± SD of six independent observations.

5.3.5: Physiological effect of genomic integration on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

In presence of 50 mM glucose, genomic integration showed ~1.73, ~1.72 and ~2.2 fold increase in growth profile by *Bj* intPgv, *Ml* intPgv and *Sf* intPgv, respectively. The total glucose utilization rate at the time of pH drop remained unaffected and the total amount of glucose consumed at the time of pH drop showed ~1.15, ~1.10 and ~1.4 fold decrease in *Bj* intPgv, *Ml* intPgv and *Sf* intPgv respectively, and Specific Glucose utilization Rate Q_{Glc} (g.g $dcw^{-1}.h^{-1}$) decreased ~1.6, ~2.1 and ~1.9 fold. Increase in enzyme activity improved the biomass yield by ~1.5, ~2.1 and ~2.1 fold in the integrants of *B. japonicum, M. loti* and *S. fredii,* respectively, compared to native *B. japonicum, M. loti* and *S. fredii* (**Table 5.3**).

***Rhizobium* Strains**	**Specific Growth Rate $K(h^{-1})$ *a***	**Total Glucose Utilized (mM) *b***	**Glucose Consumed (mM) *b***	**Biomass Yield $Y_{dcw/Glc}$ (g/g) *a***	**Specific Glucose Utilization Rate Q_{Glc} ($g.g\ dcw^{-1}.h^{-1}$) *a***
Bj	0.186 ±0.03	46.20 ±0.2	38.23 ±1.33	1.78 ±0.14	0.14 ±0.01
Bj intPgv,	0.417 ±0.01	49.37 ±0.12	20.80 ±0.63	5.18 ±0.81	0.05 ± 0.001
Ml	0.221 ±0.03	45.91 ±0.64	37.07 ±0.55	1.36 ±0.26	0.19 ±0.04
Ml intPgv	0.471 ±0.02	49.60 ±0.17	24.47 ±0.37	3.13 ±0.07	0.08 ± 0.001
Sf	0.260 ±0.02	46.10 ±0.42	37.17 ±0.55	1.85 ±0.1	0.15 ±0.02
Sf intPgv	0.383 ±0.01	49.57 ±0.06	18.57 ±0.35	2.99 ± 0.1	0.06 ± 0.001

Table 5.3: Physiological variables and metabolic data of *B. japonicum, M. loti* and *S. fredii* integrants grown on 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate. The results are expressed as Mean ± S.E.M of 6-10 independent observations. *a* Biomass yield $Y_{dcw/Glc}$, specific growth rate (k) and specific glucose utilization rate (Q_{Glc}) were determined from mid log phase of each experiment. *b* Total glucose utilized and glucose consumed were determined at the time of pH drop. The difference between total glucose utilized and glucose consumed is as explained in Section 2.9.3. * P<0.05, ** P<0.01 and *** P<0.001.

5.3.6: P solubilization and organic acid by *B. japonicum, M. loti* and *S. fredii* integrants in 50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.

There was significant increase in release of P by ~8.28, ~6.79 and ~7.86 fold by *Bj* intPgv, *Ml* intPgv and *Sf* intPgv, respectively, compared to wild type when grown on 50 mM Tris-Cl Buffer pH 8 containing 50 mM glucose containing Rock Phosphate 1mg/ml (**Fig. 5.9**).

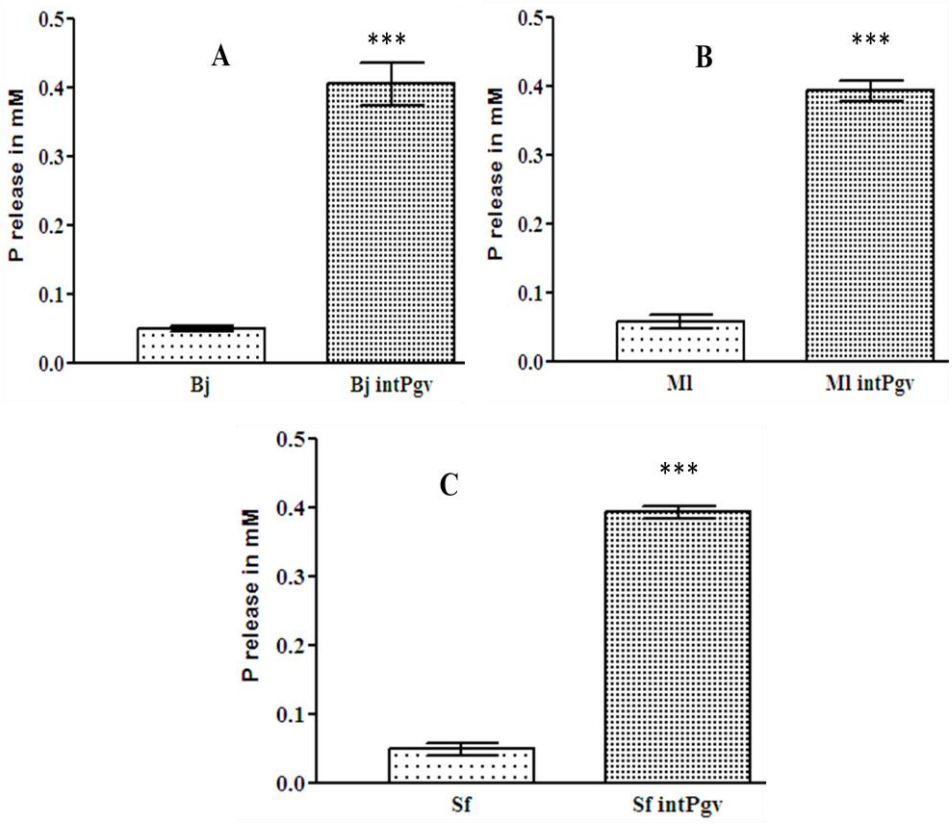


Fig. 5.8: P Solubilization by *Rhizobium* integrants.(A)- ▭, *Bj*, ▭, *Bj*. intPgv) (**B**)- ▭, *Ml*, ▭, *Ml.* intPgv and (**C**) ▭, *Sf*, ▭, *Sf.* intPgv; on **50 mM Tris-Cl buffer pH 8 and 50 mM glucose containing rock phosphate.** Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

Extracellular medium of integrants of *B. japonicum, M. loti* and *S. fredii* had 3.32, 2.81 and 3.90 folds higher amounts of 2-KGA, respectively, whereas levels of GA acid were 3.28, 3.07 and 2.64 folds higher compared to native and controls (**Table 5.4**). *Bj* intPgv, *Ml* intPgv and *Sf* intPgv also secreted ~3.4 µM , ~3.64 µM and ~4.26 µM PQQ, there is ~2 fold decrease compared to pJNK6 transformants (**Fig. 5.9**).

Rhizobium Strain	**GA mM**	**2-KGA mM**	**Specific Yield GA $Y_{C/G}$**	**Specific Yield 2-KGA $Y_{C/G}$**
Bj	6.26 ± 0.23	2.37 ± 0.47	1.39 ± 0.12	0.49 ± 0.09
Bj intPgv	20.69 ± .27***	7.87 ± .20***	1.04 ± 0.06	0.40 ± 0.12
Ml	6.53 ± 0.18	2.60 ± 0.20	1.01 ± 0.21	0.40 ± 0.1
Ml intPgv	18.40 ± 0. 4***	6.73 ± .25***	0.88 ± 0.02	0.31 ± 0.03
Sf	6.760 ± 0.10	2.20 ± 0.15	1.59 ± 0.05	0.54 ± 0.10
Sf intPgv	22.41 ± .42***	8.6 ± 0.23***	1.22 ± 0.03	0.46 ± 0.02

Table 5.4: Organic acid secretion and organic acid yield of *B. japonicum*, *M. loti* and *S. fredii pqq* integrant grown on TRP medium 100mM Tris-Cl buffer pH 8 and 50mM Glucose containing Rock Phosphate 1mg/ml. The results are expressed as Mean ± S.E.M of six independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

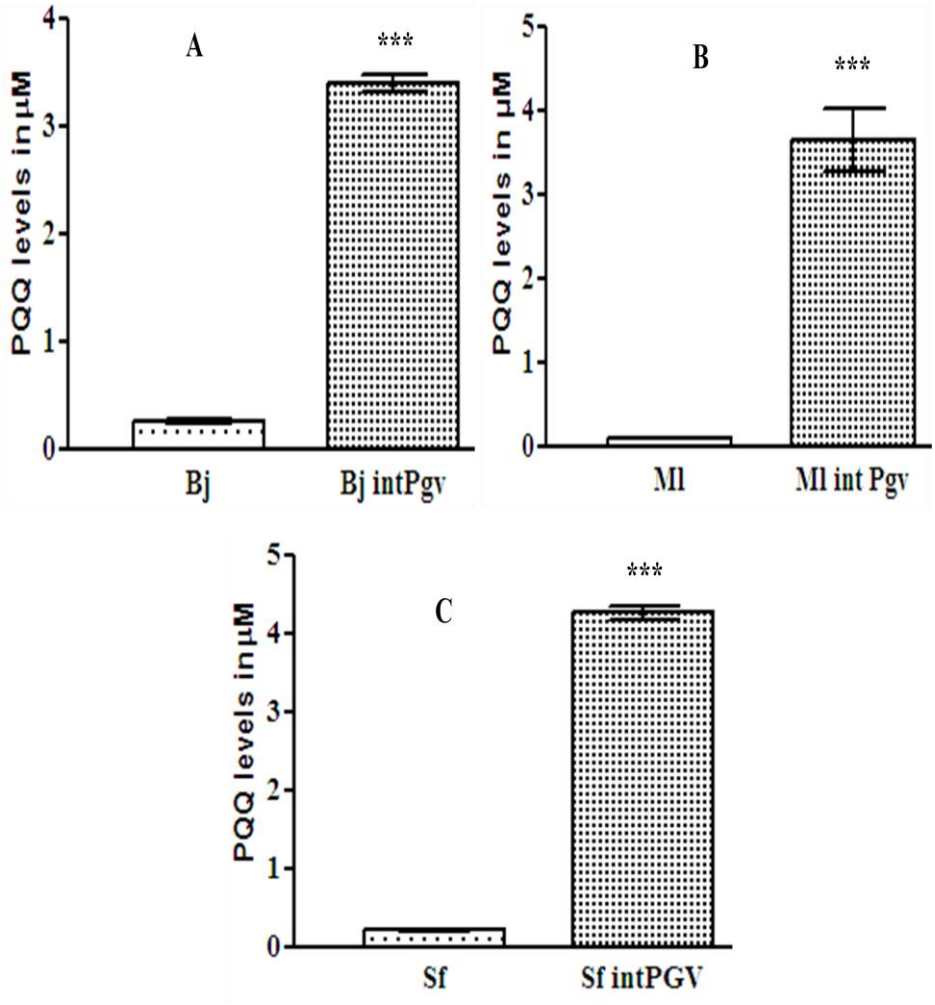


Fig. 5.9: PQQ secreted by *Rhizobium* integrants. (A)-▭, *Bj*,▭, *Bj*. intPgv) (**B**)-▭, *Ml*, ▭, *Ml.* intPgv and (**C**)▭, *Sf*, ▭, *Sf.* intPgv; on **50 mM glucose in M9 medium** Results are expressed as Mean ± S.E.M of 4-6 independent observations. * P<0.05, ** P<0.01 and *** P<0.001.

5.4: DISCUSSION

High-level expression of a target gene may be achieved by employing a high copy number vector. However, gene expression systems based on high copy number vectors have a number of drawbacks, such as , instability of recombinant plasmids, loss of the desired traits, formation of insoluble protein aggregates , decreased growth rate, metabolic perturbations (Han. 2004). Hence, genetic manipulations need to be directed towards chromosomal integration as it would lead not only to increased stability but also decrease the metabolic load caused by the presence of the plasmids or to nullify the adverse effect on the host metabolism. The present study describes the genomic integration of *A. calcoaceticus pqq* gene cluster and *P. putida gad* operon, *V. hemoglobin* (*vgb*) and *egfp* using Tn7 based integration system at *att* site in *B. japonicum, M. loti* and *S. fredii* strains and compares its effect to plasmid based expression on growth amd MPS ability.

In our study, *pqq* cluster under constitutive *lac* promoter and *gad* operon with its own promoter were integrated in to genome of *B. japonicum, M. loti* and *S. fredii* NGR 234. GADH and GDH activities in M9 medium were decreased by ~ 2 and ~ 1.5 fold, respectively compared to plasmid overexpressed strain. It appears that higher level of overexpression of genes by more copy number of plasmid is more favourable for growth and P solubilization in laboratory condition than single copy on chromosome.

Integrants showed increased PSI on PVK plates compared to MPS phenotype on TRP medium containing 50 mM Tris pH 8.0 while corresponding plasmid transformants showed phenotype even on 100 mM Tris pH 8.0 medium. Hence integrants by releasing ~ 21 mM GA and ~ 7 mM 2-KGA, can solubilize Ca-P efficiently compared to pJNK6 transformants. This shows that multiple copies of pUCPM18 does not have any effect on metabolic load in *Rhizobium* strains.

Specific growth rate, biomass yield increased in genomic integrant of *Rhizobium* strains, which was contrary to *E. asburaie* PSI3 results where growth and gluconic acid secretion decreased (Sharma et al., 2011).

Activity of *A .calcoaceticus* GDH in micro-aerobic condition on glucose-acetate mixture was lost due to loss of PQQ activity, which was restored by addition

of oxygen (Van Schei et al., 1988). In this study along with *pqq* cluster and *gad* operon, *vgb* is integrated in *Rhizobium* genome to improve the recombinant protein production and better survival of host under microaerobic conditions (Frey and Kallio, 2003). Effect of *vgb* on growth, GA and 2-KGA secretion and MPS ability in microaerobic condition is remains to be investigated.

Genetic modification either by plasmid or chromosomal integration resulted in conferring MPS and PGPR attributes to *Rhizobium* strains. This is a novel study as till now, role of P in BNF and effect on growth and yield of plants were studied by co innoculation of *Rhizobium* species with PSMs. It would be interesting to see effect of P solubilizer - Nitrogen fixer *Rhizobium* on plant growth.

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.

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^oC, 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).

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**).

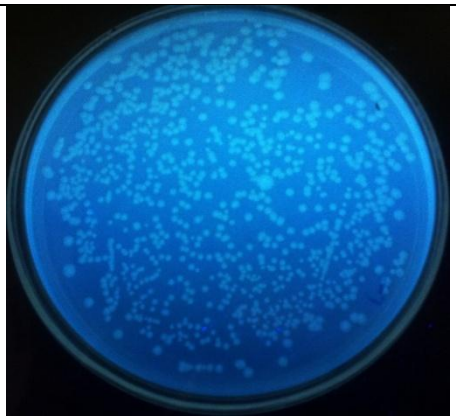
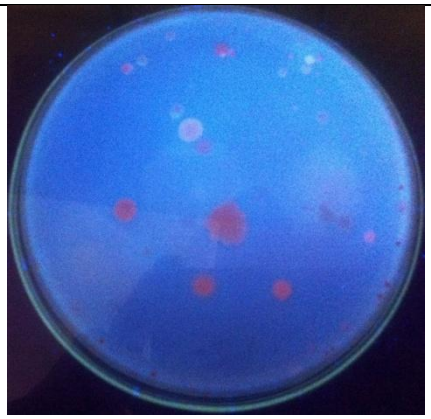
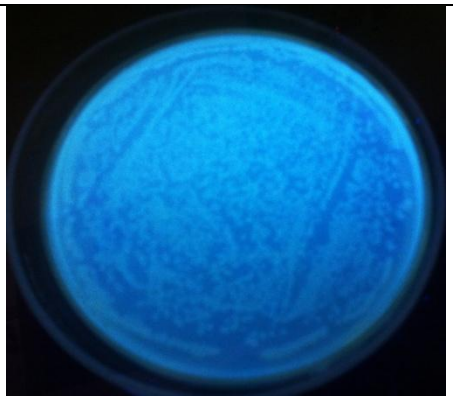
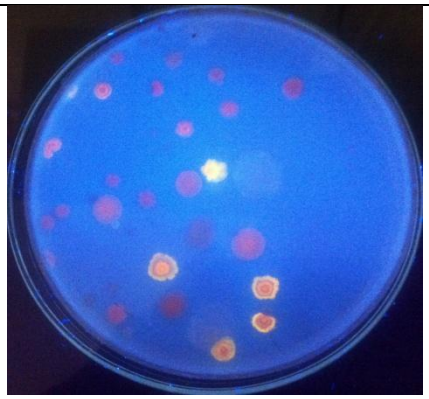
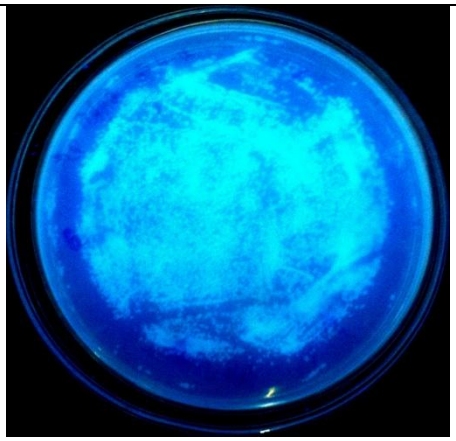
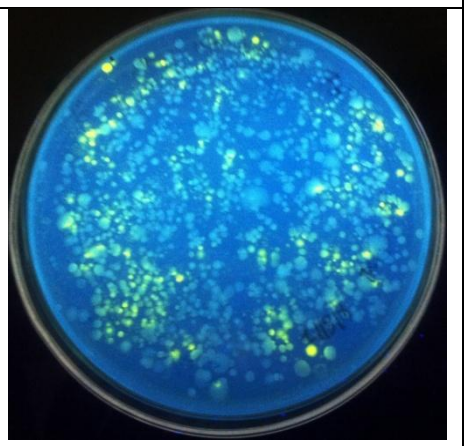
Bacteria isolated from nodules of mung bean plant	**Bacteria isolated from experimental soil**
	
Control	**Control**
	
Native	**Native**
	
Integrant	**Integrant**

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**).

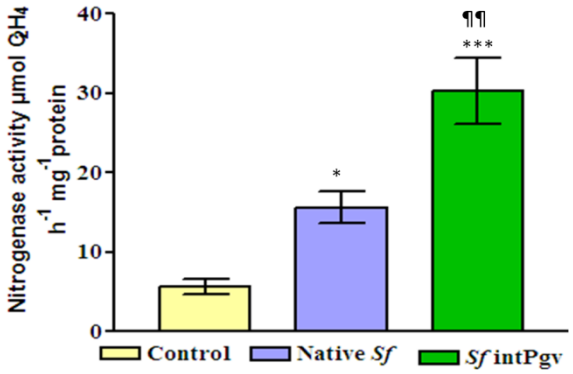


Fig. 6.2: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv on nitrogenase activity of mung bean at 45 Days after sowing. * represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations.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

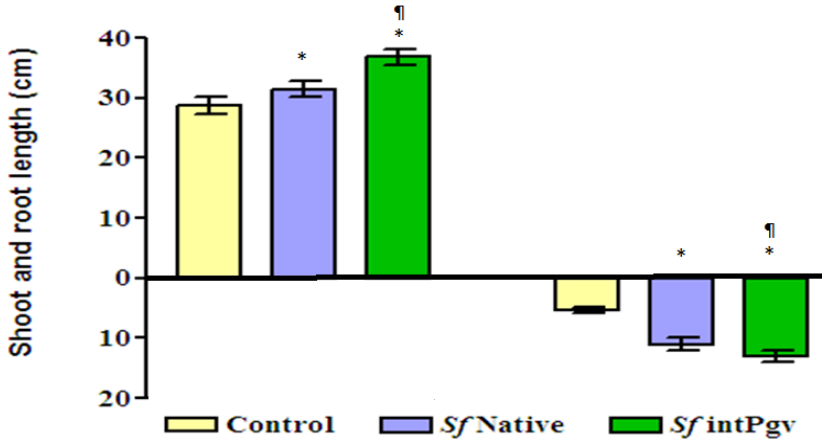


Fig. 6.3: Effect of *S. fredii* genomic integrant on shoot length and root length of mung bean at 20 Days after sowing.

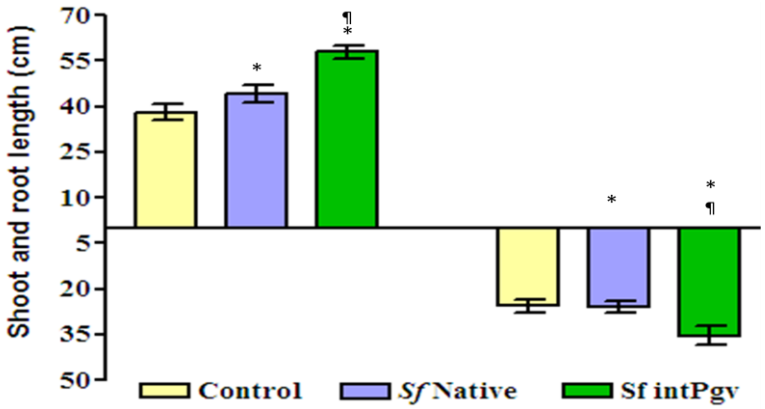


Fig.6.4: Effect of *S. fredii* genomic integrant on shoot length and root length of mung bean at 45 Days after sowing.

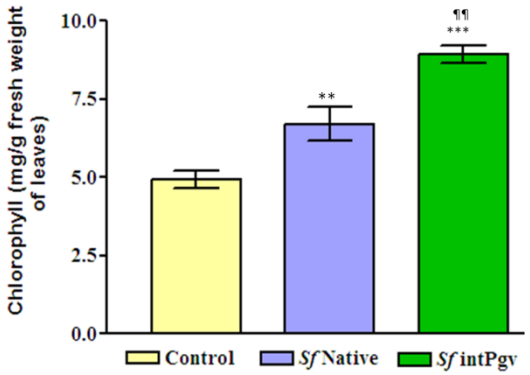


Fig. 6.5: Effect of *S. fredii NGR 234* genomic integrant on chlorophyll content of mung bean at 45 Days after sowing. * Represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations. ***, P<0.001,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

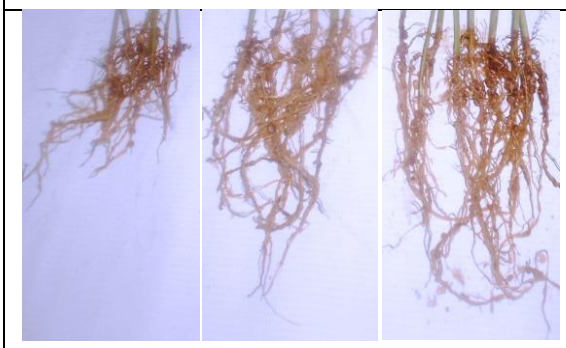
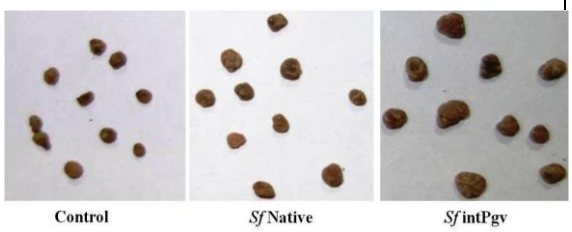
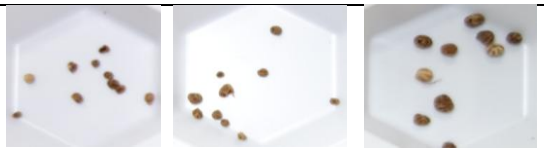
Details of 20 Days old Plants	Parameter	Control	Native	Integrant
	Plant fresh weight in gm	2.20 ± 0.26	3.72 ± 0.10	5.23± 0.51
	No of leaves	6.33 ± 1.52	10.00 ±1.73	17.66± 1. 52
	Leaf area cm^2	17.33 ±2.08	21.66 ± 2.08	25.66 ± 1.5
	No of nodules /plant	23.33 ±3.51	31.66 ± 3.08	74.00 ±2.00
	Weight of 10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

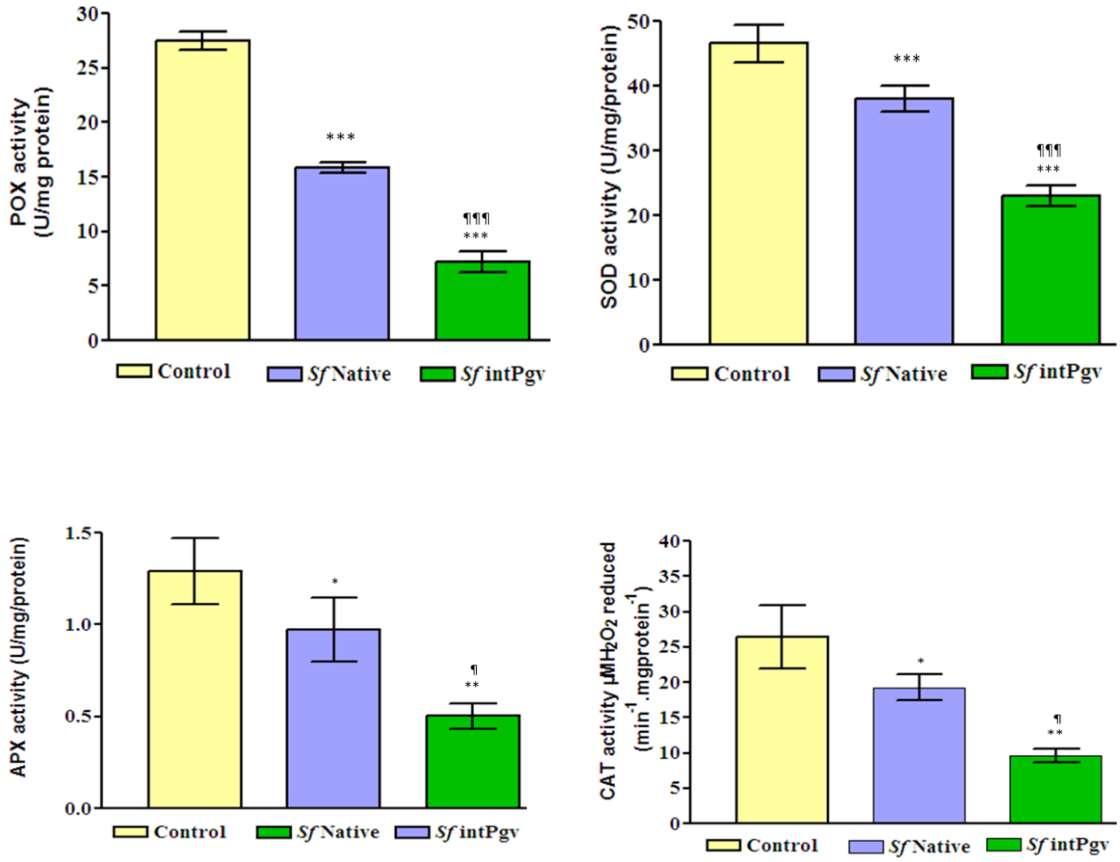


Fig. 6.9: Effect of *S. fredii* NGR234 genomic integrant, *Sf* intPgv on enzyme activities of mung bean at 45 Days after sowing. * represents comparison with the control and ¶ represents comparison of the integrant with the native. The values are depicted as Mean ± S.E.M of 3 independent observations.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

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.

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