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 renewed attention for their 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.; *Enterolobium* 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**).

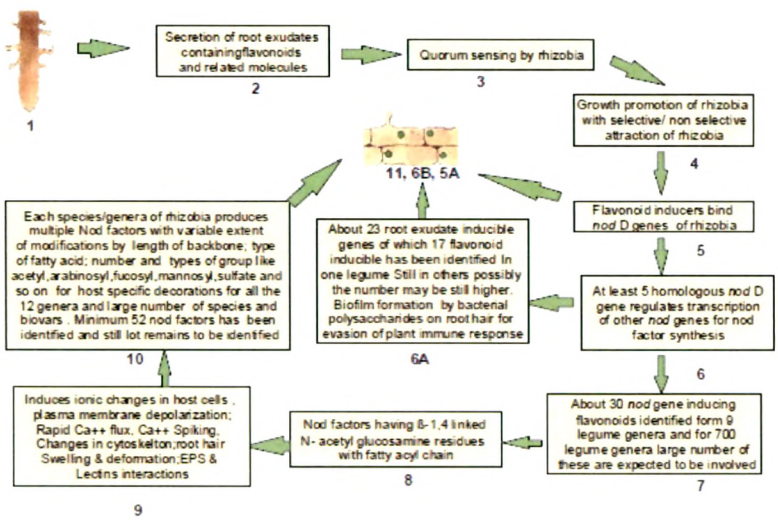

Fig. 1.1 Flavonoid, Nod factors and signal molecules exchange during nodule development.

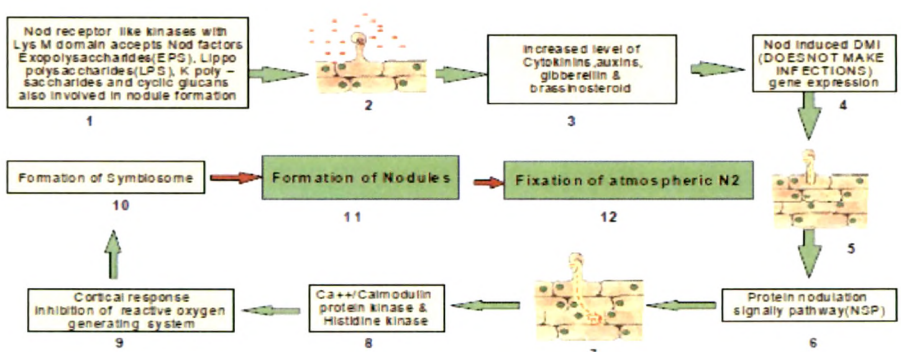

Fig. 1.2: EPS, phytohormones, endo-duplication, differentiation and development of functional nodules.

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**).

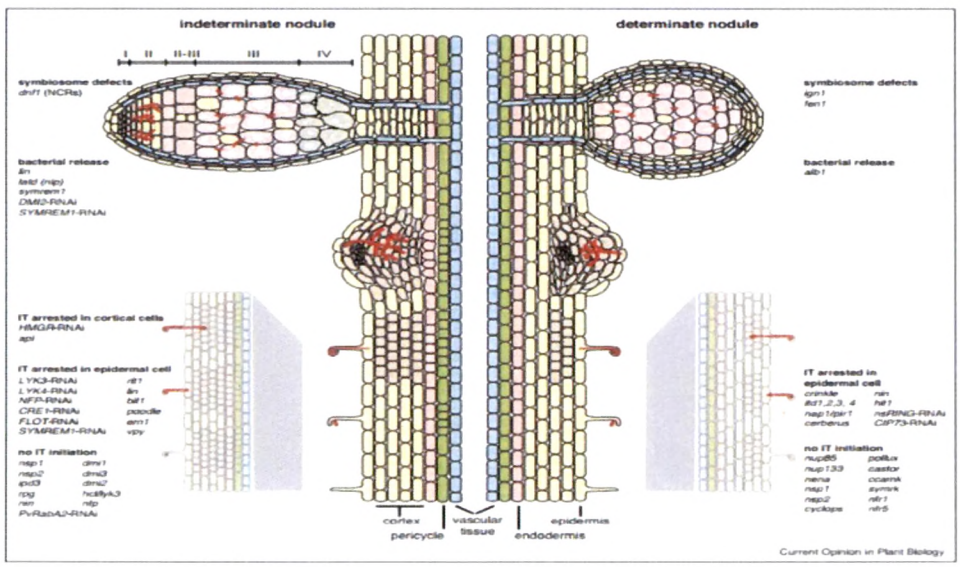

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Fig. 1.3: Development of determinate and indeterminate root nodules (Popp and Ott, 2011).

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).**

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

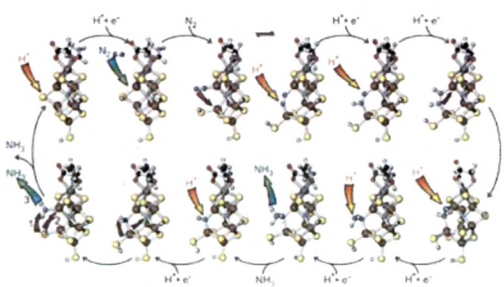

Fig. 1.4: Nitrogenase enzyme mechanism.

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**.

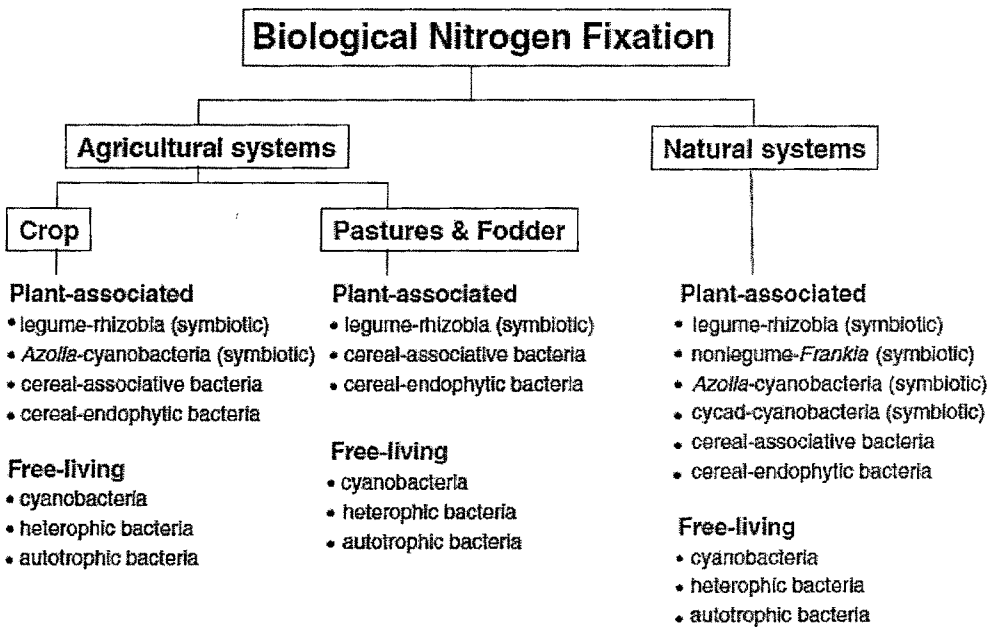

Fig. 1.5: The different N_2-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems (Herridge et al., 2008).

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 Rhizobacteria (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).**

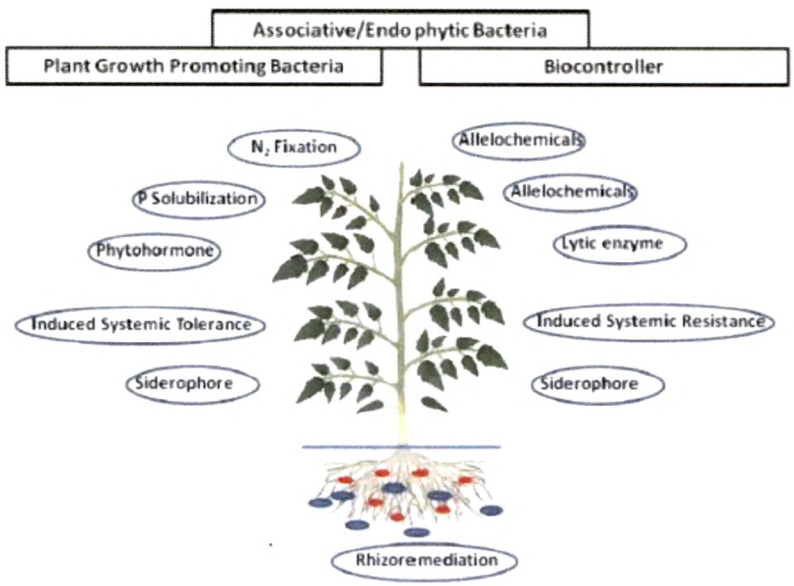

Fig.1.6: Properties of associative/endophytic bacteria for plant growth improvement (Jha et al., 2012).

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). (ref. missing in References section)

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).

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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

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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

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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).

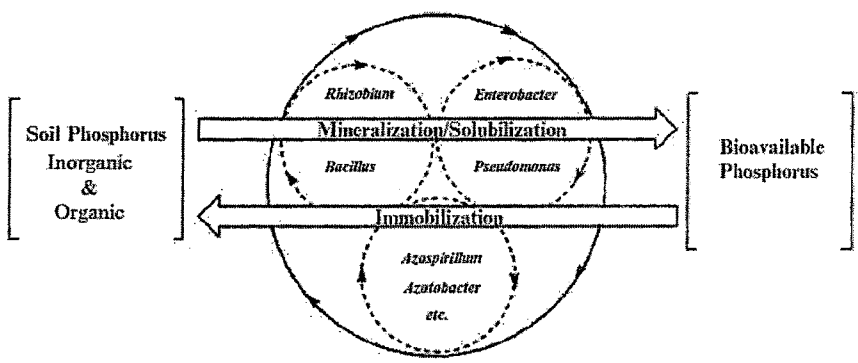

Fig.1.7: Schematic diagram of soil phosphorus mobilization and immobilization by bacteria (Khan et al., 2009)

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**).

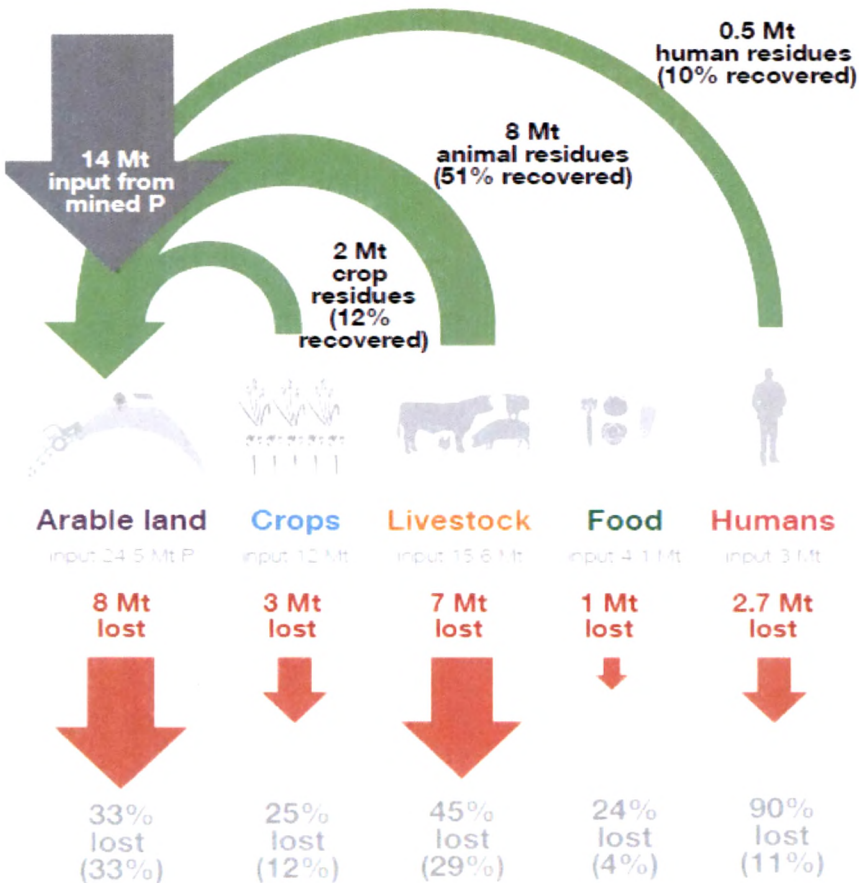

Fig. 1.8: Simplified cycle of phosphorus in agriculture (based on data from Cordell et al., 2009 and 2011). Red arrows represent losses into water systems ultimately, and green arrows represent current recoveries into arable land from the different subsystems. The percentages under the red arrows represent 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.

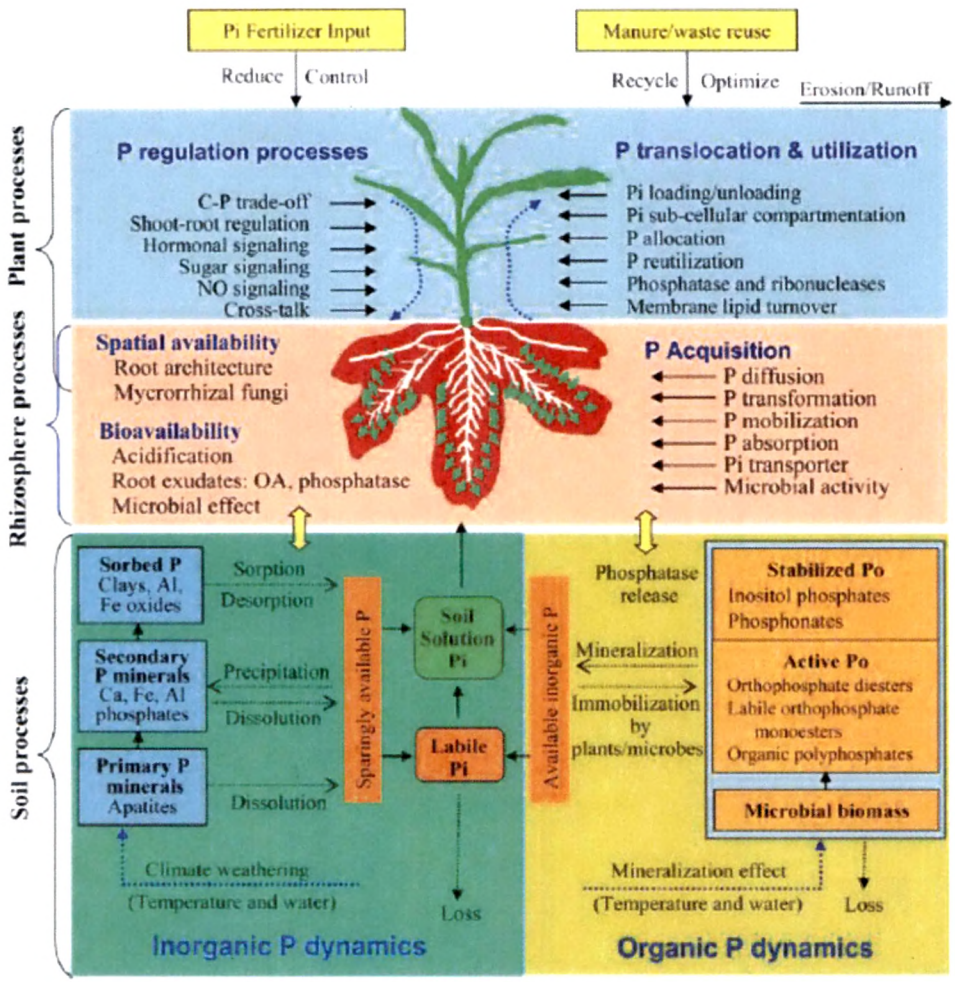

Fig. 1.9: P dynamics in the soil/rhizosphere-plant continuum C-P, Carbon- P; NO, nitric oxide; OA, organic acids (Shen et al., 2011).

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.

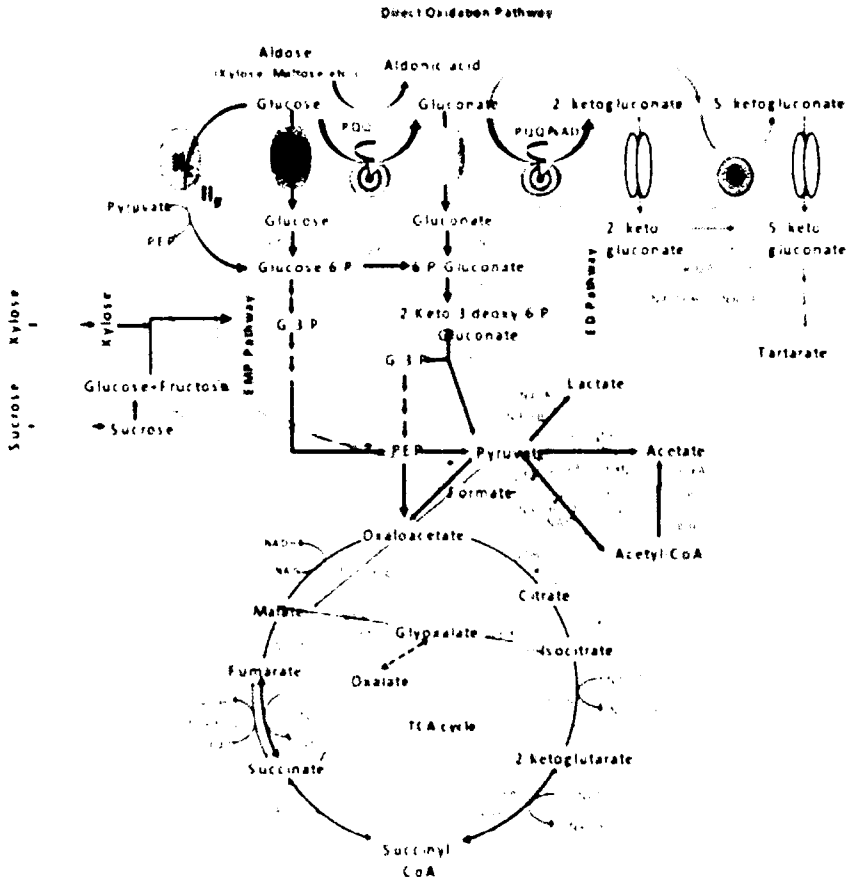

Fig. 1.10: Pathways and enzymes involved in organic acid biosynthesis by rhizobacteria (Archana et al., 2012). The organic acids secreted are depicted in boxes. The diagram depicts a comprehensive set of pathways – all may not be present in any given organism. Abbreviations: GDH glucose dehydrogeanse, 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)

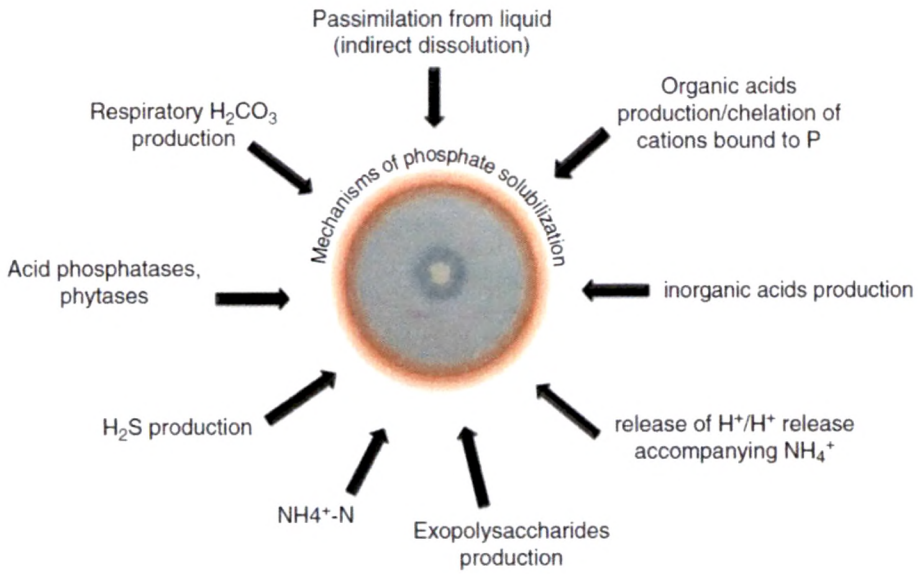

Fig. 1.11: Mechanisms of P-solubilization by phosphate solubilizing bacteria (Zaidi et al., 2009).

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).**

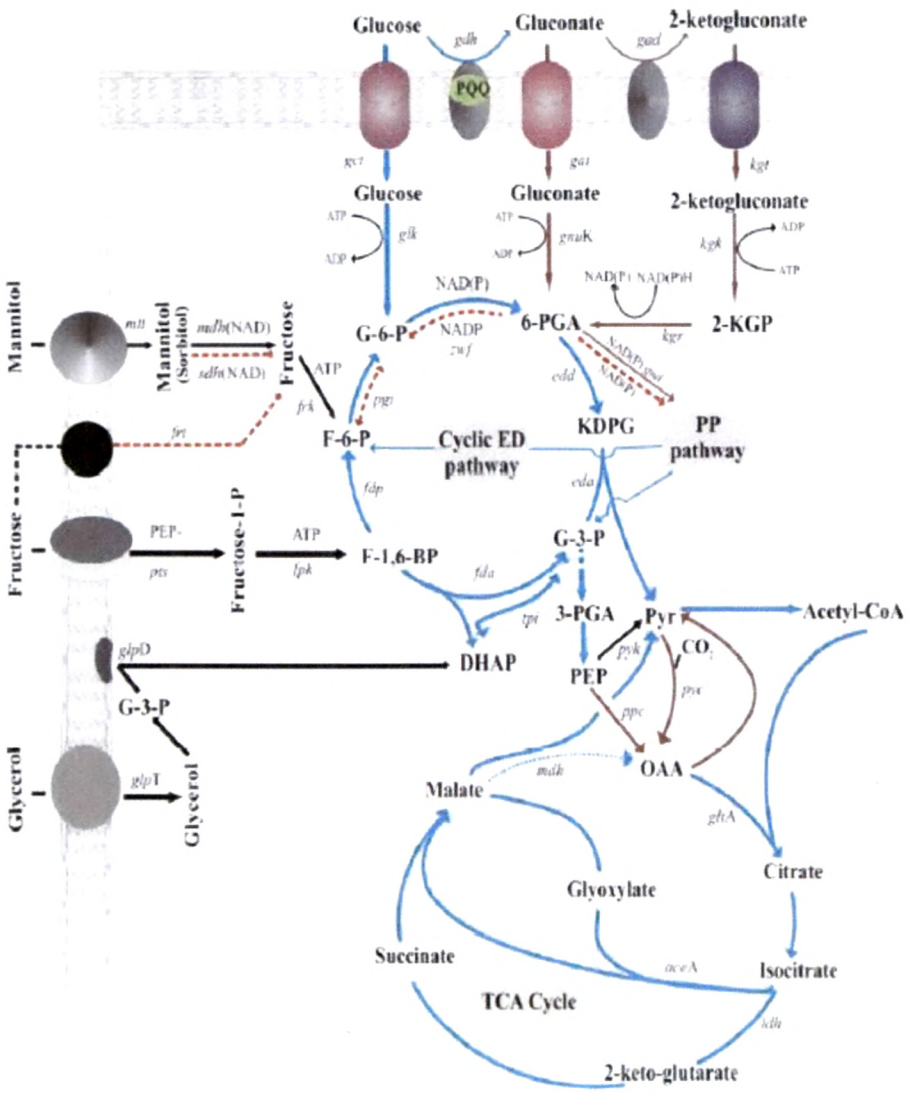

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Fig. 1.12: Carbohydrate metabolism in pseudomonads. Key to the pathway: Blue lines/arrows=Pathway operating in presence of glucose; **Bold Black arrows**=Reactions occurring when carbon source is other than glucose; Blue dashed arrow=Flux through that

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).

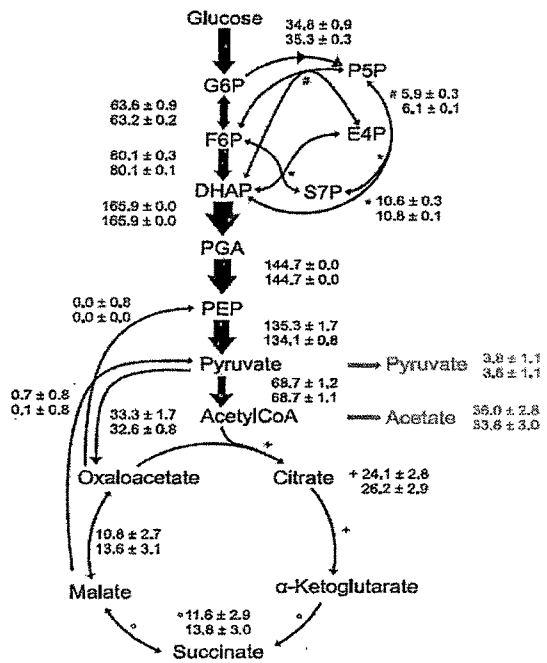

Fig. 1.13: Glucose Metabolism in *Bacillus subtilis* (Martin et al., 2011). Shown are relative flux values normalized to the glucose uptake rate of 8.2mmol g-1 h-1. Black arrows depict maximum and inner white arrows the minimum estimated flux value based on the Monte Carlo bootstrap error estimates with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

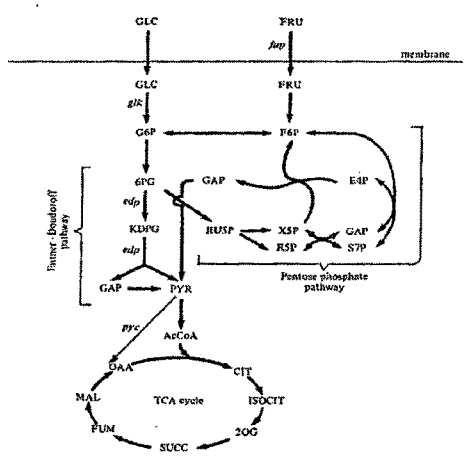

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Fig. 1.14: Pathways of glucose and fructose catabolism available to *R. trifolii* strain 7000 (Ronson and Primrose, 1979). The mutants are blocked at the steps indicated: *glk*, strains 7009, 7013 and 7039; *fup*, strain 7039; pyc, strain 7049. Strain 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-phosphogluconate; 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).

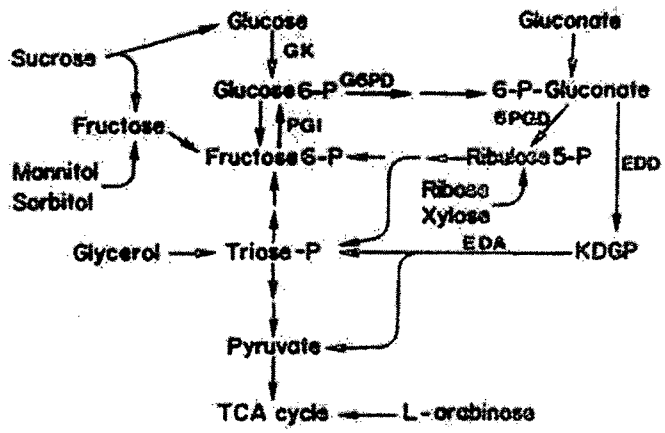

Fig. 1.15: Outline of Possible pathways of Carbohydrate metabolism in *R. meliloti* (Arias et al., 1979).

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).

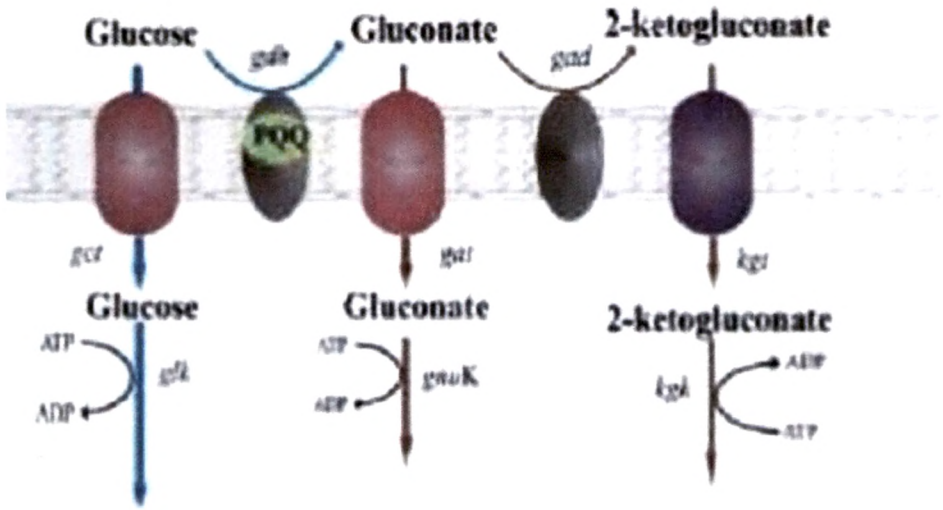

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Fig. 1.16: Direct oxidative pathway in *Pseudomonads* (Archana et al., 2012).

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).**

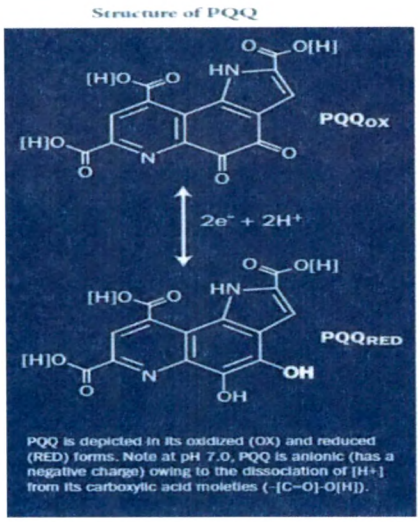


Fig. 1.17: Structure of PQQ (Rucker et al., 2009).

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

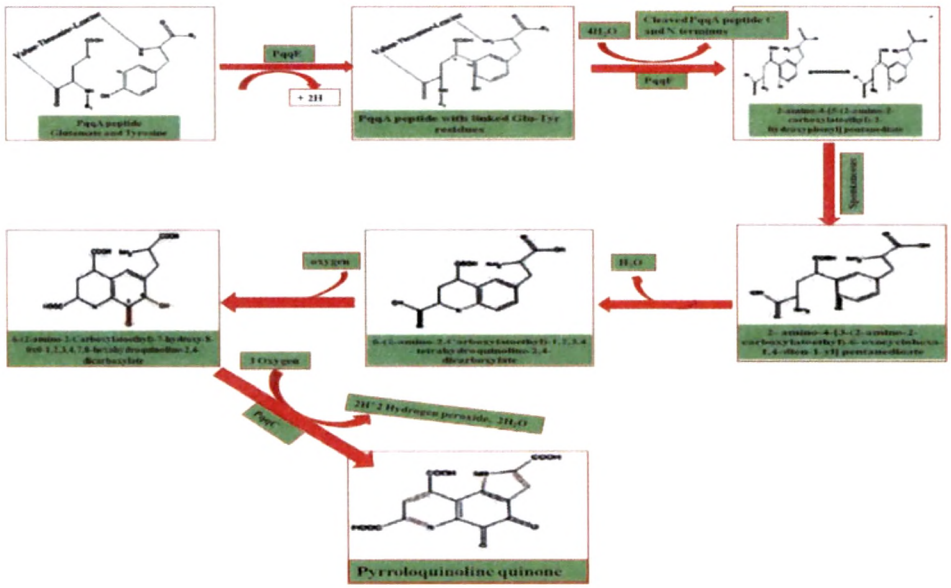
Fig. 1.18: PQQ as a redox cycling agent (Rucker et al., 2009).

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).



Improve figure

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).

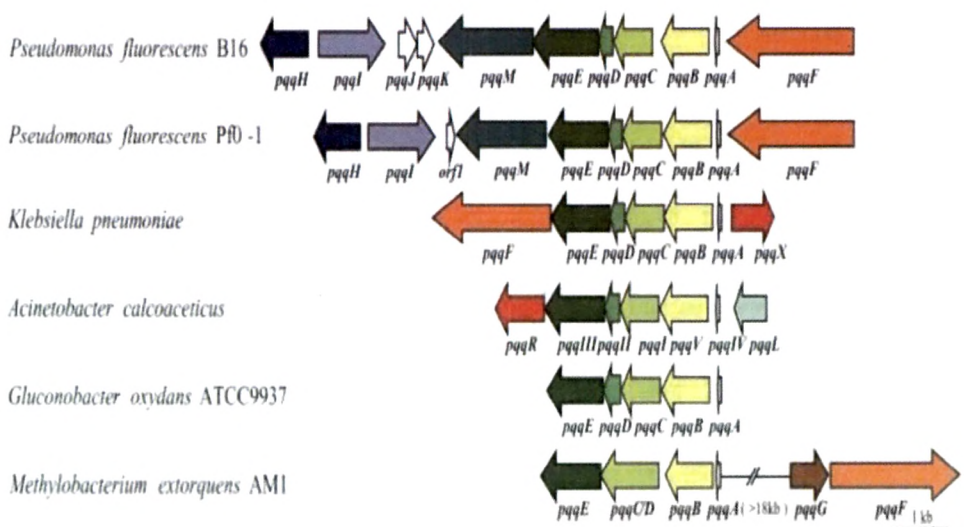


Fig. should be more sharp

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).

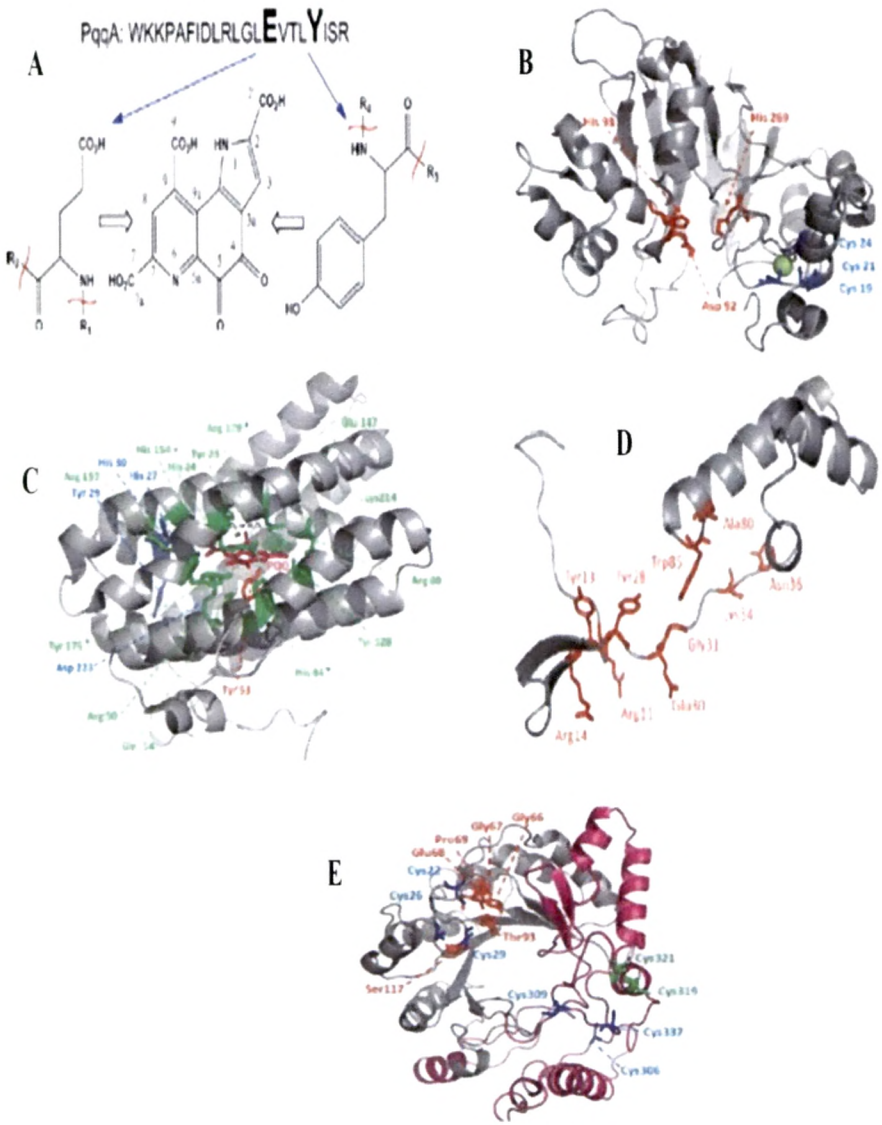


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Fig. 1.21: Structures of Pqq genes. A- PqqA, B- PqqB, C- PqqC, D- PqqD and E- PqqE (Shen et al., 2012).

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 involved 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.