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REVIEW

RHIZOBIUM – an effective and beneficial soil bacterium

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Rhizobium is a soil habitat Gram negative bacterium which is associated symbiotically with the roots of leguminous plant. The symbiosis is based on specific recognition of signal molecules produced by bacteria and its plant partners .Process of symbiosis results in biological nitrogen fixation in which nitrogen (N_2) is converted into ammonia (NH_3) and is subsequently available for plants and in turn plants provide nutrients to the bacteria .This genus of bacteria is aerobic in nature and produces acidic reaction on mineral salt medium or carbohydrates. Presently *Rhizobium* is classified into groups *Rhizobium*,*Bradyrhizobium* ,*Azorhizobium* ,*Mesorhizobium* and *Sinorhizobium* . As the plant growth promoting Rhizobacteria, *Rhizobium* promotes overall growth and development of the plant through direct and indirect mechanism. To reduce the utilization of the chemical fertilizers which disturbs the ecosystem, *Rhizobium* biofertilizers are prepared commercially. Bio-inoculant of Rhizobia can effectively improve the agricultural yield and productivity which indicate that *Rhizobium* is an effective plant growth promoting microbe.

Key words: *Rhizobium* , biological nitrogen fixation, symbiosis, PGPR, biofertilizer

INTRODUCTION

The word *Rhizobium* comes from the Greek words "rhiza" which refers to root and " bios" which refers to life .The soil contains many types of microorganisms, such as, bacteria,actinomycetes , fungi, algae etc.They are important because they affect the soil's physical,chemical and biological properties. For example, the process of decay, breakdown,and disappearance of dead plant and animals occurs due to the action of many types of microbes. Among the soil bacteria there is a unique group called Rhizobia. *Rhizobia* are Gram negative soil bacteria that adhere to and colonize the root cells of leguminous plants, including soybean and alfalfa. *Rhizobium* is soil inhabiting bacteria that form the root nodule where symbiotic biological nitrogen fixation occurs. Rhizobial inoculants are commonly applied to seeds of legume crops to ensure effective nitrogen fixation by the bacteria. The inoculants are often used together with agrochemicals besides containing essential nutrients.(Khan and Jorgensen, 2009).

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DISCOVERY

Discovered and described in 1889. R. *leguminosarum* is the type species of *Rhizobium*. *Rhizobium* has been the type genus of family Rhizobiaceae. As such it can be viewed as a representative of the genus.

HABITAT

The roots of leguminous plants is one of the important habitat of rhizobia .The habitat of the legumes are usually in soil .In the soil, *Rhizobium* are free living, motile, feeding on the remains of dead organisms. Free living *Rhizobium* cannot fix nitrogen and they have a different shape from the bacteria found in root nodules .They are regular in structure ,appearing as straight rods ,in root nodules they exists as irregular cells called bacteroids,which are often club shaped and Y shaped .

CLASSIFICATION

Rhizobium has been classified in Bergey's Manual of Determinative Bacteriology , was first published in which cataloguing of information of identifying bacteria was included .Much Revision by the American Society of Bacteriology provided such references which are followed by Bergey'sManual of systemic Bacteriology.Speciation of*Rhizobium* is based on the cross – inoculation given by the classical studies of Fred.

CROSS INOCULATION GROUPS

The basis for cross inoculation grouping lies in the ability of an isolate of *Rhizobium* to form nodules on roots of a limited species of legumes which are related to one another.Scientists have studied the matching system for many important food,forage and tree legumes .They have categorized *Rhizobium* and their legume partners into cross inoculation groups .

- Clover group: - R. *trifolii* infects and nodulates plant of genus – *Trifolium* (clover);
- Alfalfa group:- R. *meliloti* infects and nodulates the roots of *Medicago melilotus* .
- Bean group:- R. *phaseoli* infects and nodulates plants of genus *Phaseolus* .(e.g. beans);
- Pea group:- R . *leguminosarum* infects and nodulates pea , sweet pea, lentil and vetch;
- Soybean group :- R. *japonicum* infects and nodulates *Glycine,*such as soybean; and
- Cowpea group:-*Rhizobium* sp. nodulates cowpea, pegion pea, ground nut and kudz among a few others. (Table 1).

TAXONOMY

Rhizobiums is paraphyletic group that fall into two classes of Protobacteria – the Aalphabacteria and Beta proteabacteria ; most belongs to the order Rhizobiales.

They are aerobic,Gram-negative bacteria. They possess several flagella on their polar end .This allows them to move from one location to another. There are various strains of this bacteria, some of which have granules. Although they can tolerate higher temperatures of about 38^0 *C. R .leguminosarum* ideally grow in temperatures of between 20- 28^0C. Apart from various types of carbohydrates, the bacteria also uses nitrites, and ammonium salts and various amino acids, among others for growth and development. There are different strains which includes :- R. *leguminosarum biovar* viciae384; R. *legumino* USDA23701; R.*pisi* DsM30132T and R. *fabae.* CCBAU33202T. The bacteria contains 2-55% carbon and 4-5% nitrogen.

Table 1: Cross inoculation groups of *Rhizobium*

Rhizobium sp	Cross inoculation grouping	Legume Type
R. Leguminosarum	Pea group	*Pisum ,Vicia, Lens.*
R.phaseoli	Bean group	*Phaseolus.*
R.trifolii	Clover group	*Trifolium.*
R.meliloti	Alfalfa group	*Melilotus, Medicago ,Trigonella.*
R.lupini	Lupini group	*Lupinus, Orinthopus.*
R. japonicum	Soybean group	*Glycine.*
Rhizobium sp	Cowpea group	*Vigna, Arachis.*

Each of these consists of the entire legume species that will develop nodules when inoculated with *Rhizobium* obtained from any other members of the same groups. It is also possible to differentiate *Rhizobium* on the basis of growth on a defined substrate as fast growers and slow growers. *Rhizobium* is able to produce acid or alkali on YEMA (Yeast Extract Mannitol Agar) medium. Based on this criterion, the fast growing (*R. phaseoli, R. trifolii*, *R. leguminosarum*) could be grouped as acid producers. While the slow growing (*R. japonicum*, *R. lupine* and *Rhizobium* sp. could be group as non – acid producer. *Rhizobium* currently consists of 61 species belonging to 13 different genera viz., *Rhizobium , Mesorhizobium, Sinorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Methylorhizobium, Burkholdera, Cupriavidus, Devosia, Herbaspirillum, Orchobactrum,* and *Phyllobactrum.*

As per ninth edition of Bergey's Manual of Determinative Bacteriology the genus *Rhizobium* is classified into following classification –

Genus 1 : *Rhizobium : Rhizobium leguminosarum* (*Bioverstrifolii , Phaseoliviceae), R meliloti, R. loti* –fast growing , sub – polar , flagellated strains , from Lotus and *Lupinus* with string affinity for *L. corniculatus, L. densiflorus* and *Anthyllisvulneraria*. Include the fast growing strains – nodulating *Cicer, Sesbania, Leucaena*, *Mimosa* and *Lablab*.

Genus 2 :- *Bradyrhizobium* :-Slow growing polar, or sub-polar flagellated strains nodulating soybean*, Lotus uliginosus*, *L. pendulatus* and *Vigna*. Includes those slow –growing strains nodulating *Cicer, Sesbania*, *Leucaena, Mimosa, Lablab* and *Acacia.* The possibility exits that other species will eventually be defined within the genus ,but other than *B. japonicum*, the various cultures can be designated as *Bradyrhizobium* sp. (*Vigna*), *Bradyrhizobium* sp. (*Cicer*) .

Until recently, about 40 species of *Rhizobium* belonging to the seven genera of Alpha-proteobacteria have been identified and includes: *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Methylo-bacterium* (Lemaire *et al.* 2015).

PLANT GROWTH PROMOTING RHIZOBACTERIA

The beneficial, root colonizing,rhizosphere bacteria which exert a beneficial effect on plant growth have been termed as Plant Growth Promoting Rhizobacteria . They colonize the root and survive the microhabitats associated with root surface to promote plant growth and protection. They are free living, soil borne bacteria, when they are applied to seeds, soil or crops enhance the growth of plants by providing nutrients and reducing the damage from the soil pathogen. Microbes are considered powerful forces for specific enzyme mediated fundamental metabolic process. (Ahmed and Khan, 2009) . Interaction of plants with rhizosphere bacteria affects the overall crop yield and agricultural productivity. Plant growth promoting rhizobia (PGPR) help in the growth of the plants through the direct and indirect mechanisms.Direct mechanism includes biological nitrogen fixation, phosphate solubilization, phytohormones, siderophore production, hydrogen cyanide production. Indirect mechanism includes stress and biocontrol action , antibiotic production, synthesis of antifungul metabolism etc.

POTENTIALS OF *RHIZOBIUM* IN IMPROVING NITROGEN FIXATION AND YIELDS OF LEGUMES

Rhizobium spp. are considered as PGP comes in symbiosis with legumes taking advantage of nutrients from plant root exudates. When interacting with legumes, *Rhizobium* spp. help in increased plant growth through enriching nutrients by nitrogen fixation, solubilizing phosphates, and producing phytohormones, and they can increase plants protection by influencing the production of metabolites, improve plant defense by triggering systemic resistance, induced against pests and pathogens. In addition they contain useful variation to tolerate abiotic stresses such as extreme temperatures, pH, salinity and drought. Agriculture productivity is significantly affected by nitrogen and phosphorus deficiencies, which are essential for plant growth. In agricultural settings, perhaps 80% of this biologically fixed N_2 comes from symbiosis involving leguminous plants and one of the *Rhizobium* species.

These bacteria in association with legumes can fix atmospheric nitrogen. They are introduced into agricultural systems to improve soil fertility,plant growth and limit the use of chemical fertilizers.

PLANT GROWTH PROMOTION BY *RHIZOBIUM* INOCULATION

Rhizobium spp. can enhance plant growth promotion by both direct and indirect ways. Several mechanisms are involved in the plant – growth promotions by *Rhizobium* are discussed as follows:-

Biological Nitrogen Fixation *:* Nitrogen is a vital element for plant growth. It is required for synthesis of macromolecules such as amino acids, nucleic acids and chlorophyll. In agriculture, fertilization with nitrogen products is practiced to increase the production yield of food. (Xu. and Miller, 2012; Avila- OspinaL *et al.* 2014).Biological nitrogen fixation is the process of conversion of atmospheric nitrogen into ammonia .This type of process occurs though the agency of some living organisms.This process requires two important genes (nif and nix). Microbial nif genes required for nitrogen fixation are widely distributed in diverse environments.(Gaby and Buckley, 2011; Hoffman *et al.* 2013).

The biological nitrogen fixation is an energy intensive process . It is coupled to the hydrolysis of 16 molecules of ATP to reduce one molecule of N_2 (Hubble and Kidder, 2009) and is accompanied by the co-formation of one equivalent of H_2.(Chi *et al.,* 2014), so that, it can combine with hydrogen. Its reduction reaction is :

$$N_2 + 8H_2 + 16ATP \longrightarrow 2NH_3 + 2H_2 + 16ADP + 16Pi$$

The immediate electron donor is the potent reducing agent ferredoxin and the reaction is driven by hydrolysis of ATP for each electron transferred. (Fig. 1).

release of a variety of chemicals by the root cells into the soil. In response to the secretion of certain chemicals such as flavonoids and betalains by the roots, the *Rhizobium* migrates and accumulate in the soil near the roots of the legume plant. Root hairs of legume produce specific sugar binding

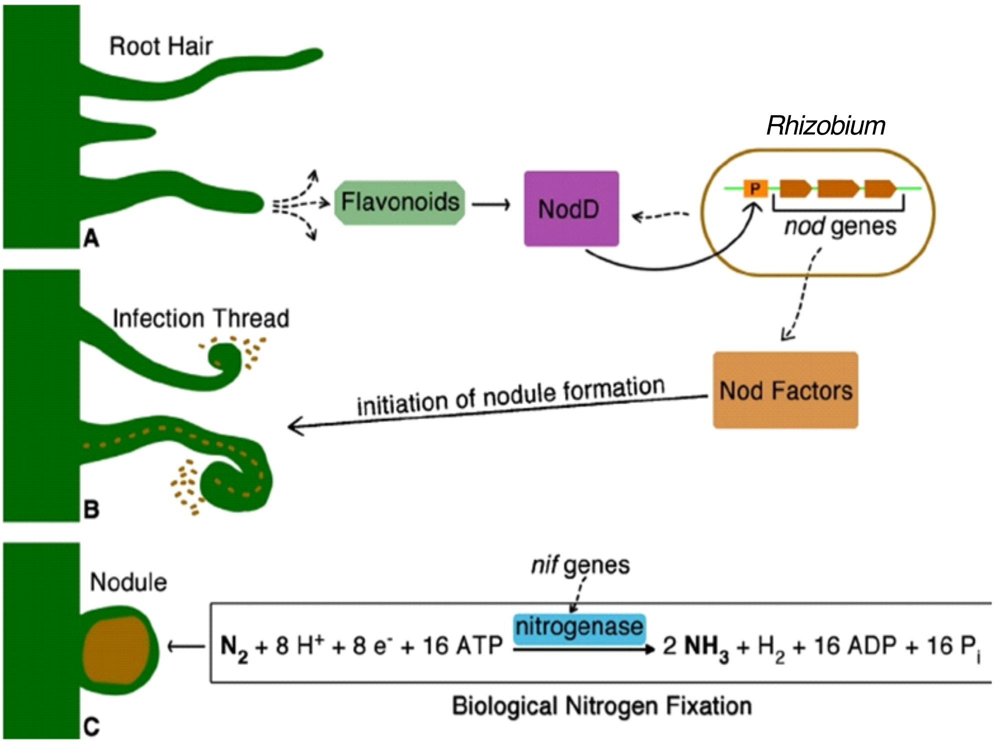

Fig. 1 : A., schematic diagram of biological nitrozen fixation

Root Nodule Formation

Nodulation is a highly host specific interaction in which specific *Rhizobium* strains infect a limited range of plant hosts . Nitrogen fixing nodules are formed by interactions of *Rhizobium* and legume host plants. Sets of genes in the bacteria control different aspects of nodulation process. One *Rhizobium* strain can infect certain species of legumes but not others .e.g, the pea is the host plant to *Rhizobium leguminosarum biovar viciae,* whereas clover acts as host to *R. leguminosarum biovar trifolii*. Specificity gene determine which *Rhizobium* strain infects which legume. Evan if a strain is able to infect a legume, the nodules formed may not be able to fix nitrogen. Such *Rhizobium* spp. are termed as ineffective. Effectiveness is governed by a different sets of genes in the bacteria from specificity genes. Nod genes direct the various stages of nodulation .The initial interaction between the host plant and free living *Rhizobium* is the proteins called as lectins . These lectins are activated by Nod factors.

Structure of Nod Factors

The chemical structures of Nod factors produced by 730 strains of *Rhizobium* have been studied. All the Nod factors produced by *Rhizobium* consists of oligosaccharide backbone beta – 1, 4 linked N-acetyl D- glucosamine. A fatty acyl chain group is always attached to the nitrogen of non-reducing saccharide .Because of resemblance of the oligosaccharide backbone to the fragment of chitin, Nod factors are also called Lipo – Chitin – Oligosaccharide (Fig. 2).

PROCESS OF BIOLOGICAL NITROGEN FIXATION

By the help of Nod factor *Rhizobium* spp. produce root hair curling factor that causes deformation and

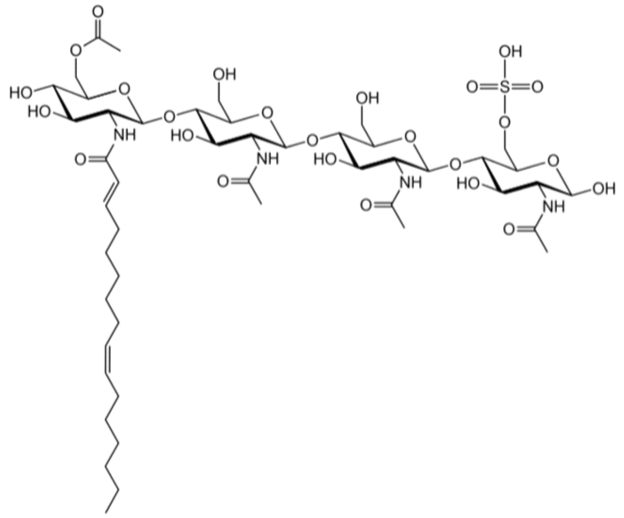

Fig. 2 : The structure of the major Nod factor produced by *Sinorhizobium meliloti*

assume nodular form.The growth and the development of a indole- acetic acid by the bacterial cells and with the entry of bacterial cells into host cells, bacterial cellular components stimulate host genome where globins are expressed. The globin protein produced in leguminous root nodules is called leghaemoglobin. (Fig. 3).

BIOCHEMISTRY OF NITROGEN FIXATION

Biological nitrogen fixation occurs in the presence of the enzyme nitrogenase (Wagner, 2011) which is found inside the nitrogen fixing prokaryote. This enzyme consist of two components –dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein). The former is a 240 kDa heterotetramer that binds N_2 and holds it while it is being reduced

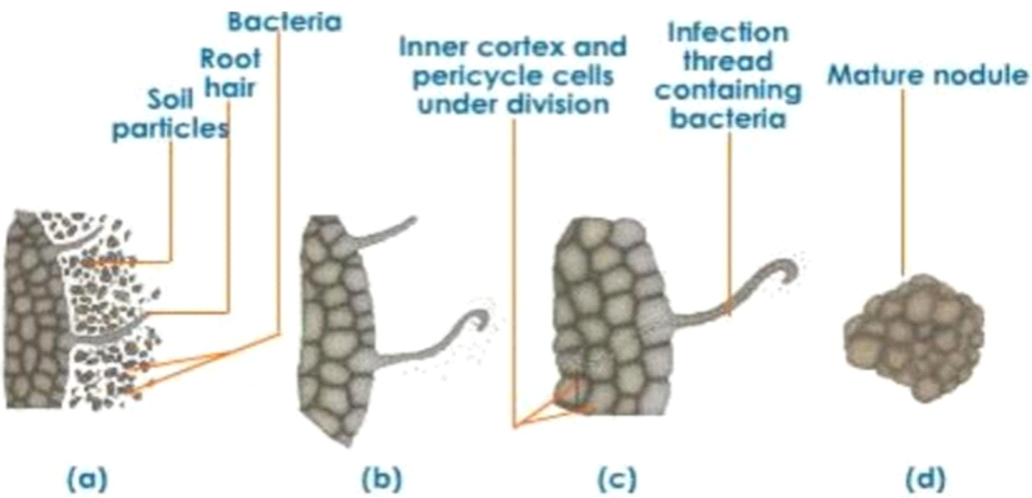

Fig. 3 : Formation of infection thread and root nodule

(a) Development of root nodules in soyabean
(b) Rhizobium bacteria contact a susceptible root hair
(c) Infection thread carrying bacteria
(d) A mature nodule complete with vascular tissues continuous with those of root

twisting of root hair.The root hair gets penetrated by a large number of *Rhizobium* which collectively convert into an infection thread .The infection thread intrudes,settles and liberates its contents in a cortical cell which is usually a polyploid .The bacteria are liberated either individually or in a small groups enclosed by a membrane , these are known as bacteroids . With the entry of *Rhizobium* cells into pericyclic cells, if the host cell is a tetraploid cell the cell, undergoes transformation into actively dividing cell. Then the infected tetraploid cells divide and redivide to produce a mass of cells which

and the latter is a 64 kDa homodimer that provides dinitrogenase with high energy electron (Fig. 4). The mechanisms proceeds *via* a series of protonation and reduction steps wherein the Fe-Mo-Co active side hydrogenates the N_2 substrate. (Hoffman *et al.* 2013).

BIOLOGICAL CONTROL OF PLANT DISEASE

In addition to their plant growth promoting effects, *Rhizobium* spp. have been increasingly associated with disease suppressive effects and which are achieved by two different mechanisms:- antagonism of pest and pathogens, and stimulation of plant – host defenses.

by the production of toxic compounds. Early work has allowed the characterization of antimicrobial activities related to extracellular compounds of *Rhizobium* spp., such as Trifolitoxin indicating that antibiosis may be part of their reported biocontrol

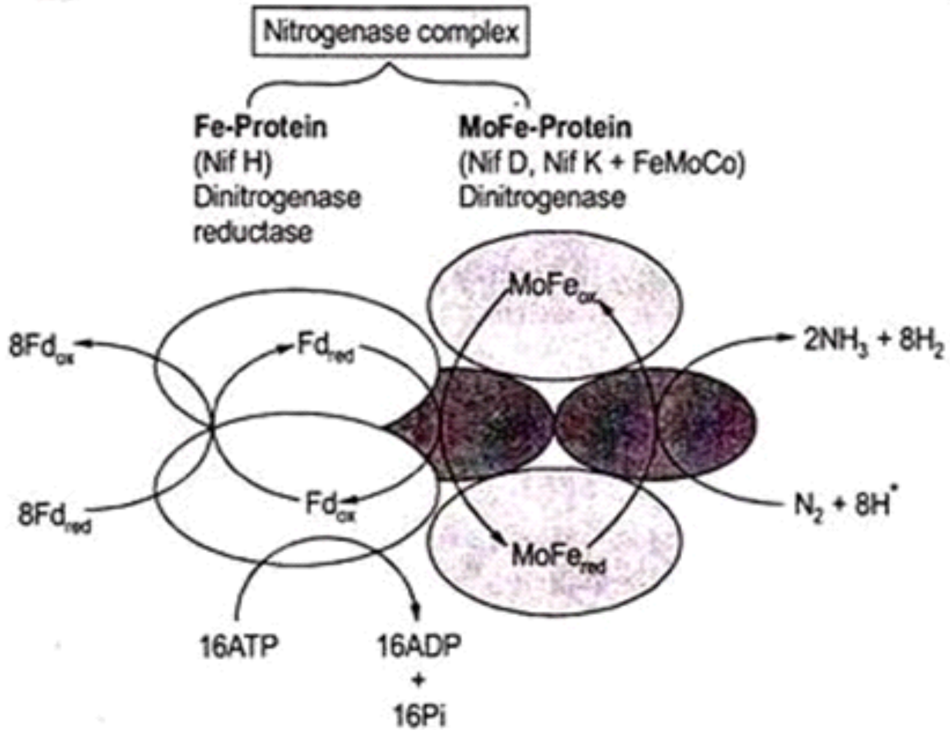

Fig : 4: Structure of Nitrogeanase complex and electron flow through the enzyme complex.The electrons are transferred from reduced ferredoxin or flavodoxin to Fe – protein component which gets reduced .From reduced Fe –protein , the electrons are given to MoFe- protein component which in turn gets reduced and is accompanied by hydrolysis of ATP into ADP and inorganic phosphate. Two ATP molecules are required per electron transferred during this process.From reduced MoFe – protein ,the electrons are finally transferred to molecular nitrogen and 8 photons, so that two ammonia and one hydrogen molecule are produced.

Antagonism of pest and pathogen population by *Rhizobium* spp. takes several forms. There is evidence ,that a strain of *Bradyrhizobium japonicum* can cause up to a 75% decrease in sporulation of *Phytophthora megaspora*, 65% in *Pythium ultimum*, 47% in *Fusarium oxysporium*, 35% in *Ascochytaimperfecta* (Tunc. Protection of soy bean from severe *Phytophtbora* root rot by. There is evidence ,that a strain of *Bradyrhizobium japonicum* can cause up to a 75% decrease in sporulation of *Phytophthora megaspora*, 65% in *Pythium ultimum*, 47% in *Fusarium oxysporum*, and 35% in *Ascochyta imperfecta*.

These findings suggest that only one bacterial strains will control a population of a multitude of pathogenic strains, thus potentially providing bioprotection for the host plant. It has been found that growth inhibition of plant pathogenis ensured efficacy. In addition to pea nodulation , inoculation with *Rhizobium* significantly protect pea against parasitic plant infection. Induced resistance in inoculated peas was characterized by reduction in seed germination of radicle growth, parasite attachment to pea roots, and finally tuber growth blockage on host roots.

INDUCTION OF PLANT DEFENSE BY *RHIZOBIUM* AGAINST PESTS AND DISEASES

Rhizobium population may also promote plant health by stimulating the plant host. *Rhizobium* spp. indirectly stimulate the plant to activate its defense mechanism through the production of plant defense compounds (phenolics, flavonoids). Induced resistance against Orobanche in peas inoculated with some strains of *Rhyzobium* was found to be associated with significant changes in levels of the defense enzyme such as – peroxidase, polyphenol oxidase , and oxidative lipoxygenase. Some isolates of *Rhizobium* spp. activating the defense in chick pea against *Fusarium oxysporium f. ciceris* in reducing the sensitivity of the disease developed in the host plant. They showed that inoculation of chick pea plants with *Rhizobium* strains ,a few days before the attack by *Fusarium oxysporum* f. *ciceris* allows the reduction of the incidense of wilting resulting from the significant increase in the activities of several defense related enzymes, such as –peroxidase and polyphenol oxidases, resulting in the accumulation of phenolic compounds and the expression of genes related to phenylpopanoid defense.

RHIZOBIUM BIOFERTILIZER

This belongs to bacterial group and the classical example is symbiotic nitrogen fixation .It has been estimated that 40-250 kg N/ha/year is fixed by different crops by microbial activities of *Rhizobium.* Liquid formation of *Rhizobium* biofertilizers are white in colour, do not have bad smell (Table 2) and no foam formation with pH 6.8-7.5 whereas carriers biofertilizer are applied in seed inoculants form. However, lack of awareness, absence of supportive infrastructure and limited research targeting the diverse and elite strains of *Rhizobium* associated with the newly improved bean lines such as MAC constraints the utilization of biofertilizers in bean production (Ramaekers *et al.,* 2013).

Importance of Rhizobium

In Agriculture : Rhizobia bacteria play a significant role in provision of agricultural ecosystem services due to their ability to form symbiotic association with a wide range of leguminous plants that results in biological nitrogen fixation (Orrell and Bennett, 2013). Some of the rhizobia strains are reported to enhance the production of phytohormones, mineral uptake and reduce toxic effects of metals, thereby, indirectly promoting plant growth and development (Karthik *et al.* 2017) in polluted agricultural soils. Modern agriculture has shifted to the use of sustainable farming practices that are eco-friendly, efficient, and affordable to the resource-limited smallholder farmers. For instance, the use of rhizobia biofertilizers in tropical areas of the Sub-Saharan Africa (SSA) has relatively increased compared to the previous decades due to the agronomic benefits associated with biofertilizers such as yield increase, cost saving, and improved soil health (Meng *et al.* 2015).

Rhizobial Endophytes in Host and Nonhost on Plant Growth and Development : Some rhizobia can form symbiotic relationships with nonlegume species such as those of Parasponia genus (Cannabaceae). The nodulation of *Parasponia* by rhizobia suggested that molecular mechanisms for plant-bacteria cross talk may be conserved and broader than expected. *P. andersonii* (Planch) has been recently found to be nodulated by rhizobia belonging to four different genera, with variable levels in nitrogen fixation efficiency, which suggested that such nontarget legumes could be reservoir for a balance between symbiotic and commensal (opportunistic) rhizobia. The rhizobial infection and nodule formation in nonlegume crops suggest potential extension of SNF to nonlegume crops such as cereals.

Table 2 : Classification of different types of biofertilizers

Serial no	Group	Examples
1. Nitrogen fixing	Nitrogen fixing biofertilizer:-	
	a. Free living	*Azotobacter, Anabaena*
	b. Symbiotic	*Rhizobium, Frankia*
	c. Associate symbiotic	*Azospirilum*
2. Bacteria	P. solubilizing Biofertilizer	*Bacillus circulans*
Fungi		*Penicillium* sp.
3. Arbuscular mycorrhiza	P. mobilizing Biofertilizer	*Colomus* sp.
4. Ectomycorrhiza.		*Laccaria* sp.
5. Conicoid mycorrhiza		*Perizella ericae*
6. Orchid mycorrhiza		*Rhizoctonia solani*

Importance in soybean production : Many crop species including soybean are found associated with abuscular mycorrhizal fungi and *Rhizobia*. Its production must be evaluated from different perspectives including it's symbiosis with soil – microbes. (Marcela and Mohammad, 2016).The main function of nodules on soybean roots is to fix the atmospheric N by the process of symbiotic nitrogen fixation, supplying nitrogen for plant growth and seed production. It has been reported that changes in the rhizospheric bacteria and especially, *Bradyrhizobium,* during soybean growth, suggesting that the symbiosis of host plant with rhizobia may be selective.

CONCLUSION

From the facts mentioned above this can be concluded that, *Rhizobium* is one of the those bacteria,that is necessary for sustaining life on earth.This nitrogen fixing bacteria, forming nodules of legumes, grow all over the world .*Rhizobium* is the most well- known species of the group of bacteria that are engaged in symbiotic relationship with leguminous plants .They obtain their nutrients from the plants and produce nitrogen fixing root nodules through a process called Biological Nitrogen Fixation .Extensive literature survey suggests that *Rhizobium* is Gram negative ,motile ,rod shaped bacteria which contains the granules of β hydroxybutyrate .These bacteria are aerobic and can grow well on a synthetic medium, YEMA (Yeast Extract Mannitol Agar). *Rhizobium* may be classified into slow and fast growing. Presently *Rhizobium* is classified into *Rhizobium, Bradyrhizobium, Mesorhizobium, Azorhizobium, Sinorhizobium .* Extensive study of literature suggests that *Rhizobium* has been an effective PGPR (Plant Growth Promoting Rhizobacteria) and helped in plant growth promotion and crop production .It had been seen that microbial biofertilizer as an effective supplement in agriculture against utilization of chemical fertilizers. Farmers can stimulate biological nitrogen fixation by applying the correct *Rhizobium* to legume crops by a process called inoculation. Thus *Rhizobium* fix nitrogen in a usable form, for plants and the only way humans can receive nitrogen through diet from plants. Therefore, *Rhizobium* inevitably help human and animal to live reducing the requirement of chemical fertilizer.

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