

## Search for the Holy Grail: *Rhizobium*

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### ABSTRACT

*Legume-Rhizobia symbiosis is of the great ecological and agronomic importance, due to their ability to fix a large amount of atmospheric nitrogen (360 Kg of N per hectare per year). Enzyme nitrogenase plays an important role in fixation of nitrogen. Acetylene reduction assay is used to measure the nitrogenase activity. Rhizobium gets attached to root hair by lectin mediated hypothesis resulting in curling of root hair, formation of infection threads followed by critical cells division. Further bacteria also divides and redivides giving rise to bacteroids surrounded by leghaemoglobin. This leghaemoglobin binds with oxygen giving rise to oxyleghaemoglobin proving anaerobic conditions within for the fixation of nitrogen. The behaviour of some nitrogen fixing systems under severe environmental conditions such as salt stress, drought stress, acidity, alkalinity, nutrient deficiency, fertilizers, heavy metals and pesticides is reviewed. These major stress factors suppress the growth and symbiotic characteristic of most Rhizobia. However, several strains, distributed among various species of Rhizobia, are tolerant to stress effects. Specific strains of Rhizobia are required to make the nodules functional in order to carry out the process. This increases the yield of crops.*

**Keywords:** Legumes, Rhizobia, Leghaemoglobin, Stress.

### INTRODUCTION

The element nitrogen comprising about 79% of total atmospheric gases is the key element which is responsible for enhancing crop production by converting atmospheric nitrogen into plant usable form. Nitrogen can also be fixed chemically which is used as a biofertilizer in agriculture at high rates but these having some global concerns such as environmental pollution, eutrophication, plant

toxicity, ozone depletion etc. (Zhang et al., 2013). Therefore, some alternative and cheap ways were found to fulfil the need of nitrogen for crop production enhancement. Plants like Pea (*Pisum sativum* (L.)) are leguminous plants that are widely cultivated in many areas because of their high nutritive and high biomass producing values. In 1886, the nitrogen fixing function in these leguminous plants were discovered (Sheng & Jing, 2003).

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Biological Nitrogen Fixation, which is a microbiological process, converts atmospheric nitrogen into plant usable form. *Rhizobium leguminosarum* strains are present in the roots of these leguminous plants and have higher ability to fix atmospheric nitrogen. These microbial strains are successfully cultured and widely used to improve Nitrogen fixing capability in legume plants. These microorganisms are able to fix approximately 360 Kg of N/ha/year in association with different leguminous crops (Peoples et al., 1995; & Sibbal et al., 2002). A part of these strains have been made available to the host to improve crop and soil productivity.

Legume-*Rhizobium* symbiosis that depends not only on the ability of introduced strains of *Rhizobium* that combat over native *Rhizobia* for nodulation to enhance its capacity but is also influenced greatly by the genetic characters that the host plant exhibit (Gresshoff et al., 2014; & Stephen et al., 2019). Genetic characters of the host plant shows the higher efficiency to fix nitrogen. The nitrogen fixation efficacy can also be determined upon the characteristics of nodulation (Minchin et al., 1978; Gupta et al., 1983 & Sohal et al., 1993).

#### REVIEW OF LITERATURE

Hellriegel (1886) was the first to observe a kind of symbiotic relationship between legumes and a bacterium which was later named as *Bacillus radicolica* (Beijerinck, 1888) and has now been placed under the genus *Rhizobium*. These bacteria though live freely in soil and in root regions of both legumes and non-legumes but form nodules only with leguminous crop called Cross Inoculation groups (Fred et al., 1932). Further, the genetic characters of the host plants, the *Rhizobia* and agroclimatic conditions are important factors responsible for the best host cultivar-*Rhizobium* strain combination for getting maximum nitrogen fixation. Therefore, continuous selection of most effective *Rhizobium* strain for a specific host cultivar and a particular region/soil is most essential.

#### LEGUME-RHIZOBIUM SYMBIOSIS

Legume crops which fix nitrogen by means of endosymbiotic *Rhizobia* are not only the major

world source of proteins but are also known to improve soil fertility and hence help in sustainable agriculture.

The formation of nitrogen fixing nodules on legume plants is the result of highly specific cell-cell interaction between the host plant and the *Rhizobium*. Dazzo (1981) viewed bacterial attachment as a cellular recognition event during the infection of legumes by *Rhizobium*. Studies on the biochemical basis of selective attachment also suggest that the recognition process is accomplished by specific glycoprotein lectin-polysaccharide interaction on the surface of symbiont. *Rhizobia* enter the legumes through root hair or directly at the point of emergence of lateral roots by forming infection thread. The *Rhizobia* are released into the cytoplasm of the host cells, where after, infected cells differentiate into non-dividing one which form the nodules tissue. Furthermore, the *Rhizobia* differentiate into characteristic bacteroid forms. During the formation of root nodule several host genes are specifically induced (Bisseling et al., 1984; Kondorosi & Kondorosi, 1986; & Nap & Bisseling, 1990). The bacterial genes involved in nodulation are activated by phenolic compounds exuded by the plant (Firmin et al., 1986; Peters et al., 1986; & Redmond et al., 1986). The nodulation genes appear, in turn, to be responsible for the production of a low molecular weight signal molecule which initiates the plant morphogenesis that leads to the production of a nodule (Faucher et al., 1988, 1989; & Schmidt et al., 1988).

Long (1989) concluded that *Rhizobium* bacteria stimulate leguminous plants to develop root nodules, which the bacteria infect and inhabit. Ultimately, the two organisms establish metabolic cooperation: the bacteria reduce fixed molecular nitrogen into ammonia, which they export to the plant for assimilation. The plant reduces carbon dioxide into sugars during photosynthesis and translocate these to the root where the bacteria use them as fuel. The microscopic studies indicate that the plant plasma membrane envelopes bacterial cells as they emerge from

discontinuities in the infection thread wall (Mellor & Werner, 1987). The bacteria undergo limited DNA replication and division and cease both the processes. They display distinct morphology and gene expression and are referred to as bacteroids. The plant derived "peribacteroid membrane" (PBM) undergoes changes in quantity and content (Brewin et al., 1985; Mellor & Werner, 1987; & Bradley et al., 1988). The specialization of the PBM may include specific transport or permeability functions. The following figure shows release of bacteria into plant cell takes place by endocytosis with plant plasma membrane. The bacteria differentiate, as does the plant cytoplasm and the peribacteroid membrane (PBM). Nitrogen is reduced to ammonia in the bacteria and exported to the cytoplasm, where plant assimilates the ammonia into glutamine.

Santalla et al. (2001) conducted an experiment to represent symbiotic interactions between *Rhizobium leguminosarum* strains and elite cultivars of *Pisum sativum* (L.)

#### HOST CULTIVAR-RHIZOBIUM STRAIN INTERACTION

In legume-*Rhizobium* association, both partners play roles in determining overall efficiency of the nitrogen fixing system (Minchin et al., 1978 & Mytton, 1978). Sometimes, even the most efficient strain of a particular host cultivar and under a particular set of environmental conditions may fail with other cultivar (Diatlaff & Brockwell, 1976 & Singh et al., 1979) and/or under different environmental conditions (Gibson et al., 1976; & Gupta et al., 1982, 1983, 1989).

Wilson (1937) studied the interaction of 4 species of sweet clover and several strains of *R.trifolii*. He observed that some species of sweet clover inoculated with certain strains of *R.trifolii* always nodulated well and fixed nitrogen in case of one cultivar but failed in case of another. Poi and Kabi (1979) observed 8 times increase in fodder yield of barseem var. Muscoy with *R.trifolii* strain Tu-8 over uninoculated control in a study conducted both under pot and field conditions with ten strains. Nutman (1956) showed that host factors could control the amount of nitrogen fixed. In a

study conducted with 25 lines and 5 cultivars of *Medicago truncatula* inoculated with each of 16 strains of *R.meliloti* of diverse origin, cultivars of Akbar and Jamalong showed appreciable specificity with respect to their strain requirement for effective nodulation. Materon (1991) conducted experiments with 247 isolates of *R.meliloti* from soil samples collected from diverse area in West Asia were assessed to define their symbiotic characteristics and degree of host specificity on six species of *Medicago* (*M. rididula*, *M. noeara*, *M. rotata*, *M. orbicularis*, *M. truncatua*, *M. polymorpha*). Host species responses were evaluated based on nodule appearance and plant biomass. The test soils contained population of *R. meliloti* with differing ability to nodulate through specific legumes and fix N<sub>2</sub>.

Bapat et al. (1977) reported the host-Rhizobium interaction in bengal gram and grouped 50 genotypes into 6 categories according to the yield responses to inoculation. The nodulation affected significantly nodule number, shoot, weight and seed yield. The genotype differed significantly to shoot weight and yield. However, they did not show differences with regard to Rhizobium compatibility and efficiency of nitrogen fixation were observed in Chickpea (Sinha, 1978; & Gupta et al., 1982). Di Bonito et al. (1990) evaluated the competition ability for nodulation of the strains of Rhizobium spp. (*Cicer arietinum* (L.)) IC 2091, 27A2, 27A15 inoculated on different Chickpea genotypes and the effect of inoculation in a fertile neutral soil (pH 7.2). The inoculation was carried out under field conditions and the nodule occupancy evaluated the indirect immunofluorescence technique. All the strains increased the number of nodules in inoculated plants. The strain 27A15 showed a serological diversity with the native strains and a nodule occupancy from 81.4 to 100 percent in inoculated plants. A percent of cross reaction with the native strains affects the results for IC2091 and 27A2. The inoculation generally increased the grain yield production. The different strains/cultivar combinations exhibit

a significant variation for nodulation productivity.

De Jong and Phillips (1981) inoculated pea plants individually with one of 15 *R. leguminosarum* strains and grown under uniform environmental conditions in the absence of combined  $N_2$ . Differences in the effectiveness of the *Rhizobium* strains produced plants with different rates of whole plant apparent  $N_2$  fixation and N allocation, apparent photosynthesis and growth. Results from the studies showed that fixed Nitrogen interacts with leaf photosynthetic efficiency and plant growth in a manner that is dependent upon the allocation of symbiotically fixed  $N_2$ . Lie (1981) found that *Pisum sativum* ecotype fulvum forms ineffective nodules with *Rhizobium* strains, isolated from effective nodules of the cultivated pea in Europe. *Rhizobium* strains isolated from nodules of fulvum peas in Israel are fully effective in this host plant but in association with the cultivated pea they induce nodules of poor- $N_2$  fixing activity. The distribution of these fulvum specific *Rhizobium* strain is restricted to region where the fulvum pea occurs naturally. *Rhizobium* strains from the geographical regions induce mainly ineffective or partially effective nodules of fulvum plants. A wide genetic variation, with regard to symbiotic response to a standard set of *Rhizobium* strains, was demonstrated in the fulvum plants collected in Israel. Based on variation in  $N_2$ -fixation three groups of plants can be distinguished. These plants offer the possibility for the study of host-genetic control on symbiotic-nitrogen fixation. Hobbs and Mahon (1983) examined symbiotic Nitrogen-fixation in 36 plants genotype-bacterial strain combinations produced by growing six genotypes of *Pisum sativum* (L.) and six strains of *R.leguminosarum* in all combinations. Both genotype and strains had effects not only on Nitrogen-fixation but also on characters associated with plant growth and photosynthesis. However, relationship between characters differed markedly depending upon which genotype or strains were used to calculate correlation coefficients.

Genotype x Strain (G x S) interaction also affected the expression of several of the characters. Using Nitrogen fixation as an example, statistical methods analogous to those developed for the analysis of genotype x environment interactions were used to study this interaction. From this analysis, it was apparent that G x S variability was caused by differences in the magnitude of the response of the plant genotype or bacterial strains to change in the complementary symbiont with little difference in stability. Chetkova and Tikhonovich (1986) isolated three *R. leguminosarum* strains capable of nodulating resistant peas of Afghanistan from North-Western USSR soils. Main symbiotic characteristics viz. – effectivity, nodulation and competitive ability were tested. Such strains may be used in combination with plant resistant genes for the construction of highly specific plant-strain symbiotic pairs. Similarly, Bhandal et al. (1989) studied symbiotic effectiveness of four cultivars of pea (PG-3, PB-87, PB-88 and PB-23) inoculated with *R. leguminosarum* with B-164. Nodulation was found to be dependent on host cultivar genotype as B-164 interacted best with var PB-23.

Somasegaran et al. (1991) detected strong combination of host x *Bradyrhizobium* in four genotypes of *Phaseolus acutifolius* tested against eight isolates of *Bradyrhizobia* and a commercial mixed inoculant. All seven progeny tested nodulated with *Bradyrhizobium* isolates and *R. leguminosarum* bv. *phaseoli*, but there was a highly specific *Rhizobial* requirement for effective nodulation and  $N_2$  fixation. The progeny x *Rhizobial* interaction accounted for 83% of the total phenotypic variation. Two (P-6 and P-7) and five (P-1, P-2, P-3, P-4 and P-5) progeny nodulated and fixed  $N_2$  effectively with *R. leguminosarum* bv. *phaseoli* and *Bradyrhizobium* spp. respectively. The *R. leguminosarum* bv. *phaseoli* progeny symbiosis had a greater  $N_2$ -fixing potential than the *Bradyrhizobium* isolate-progeny symbiosis. In a soil (oxisol) test, progeny P-6 and P-7 showed significant response to inoculation with *R.*

leguminosarum bv. phaseoli strain R. leguminosarum bv. phaseoli. TAL 182 was the most competitive strain occupying 84% of the nodules in both the progeny. The genotype Puebla 152 which nodulates profusely in Columbia and has been used as a parent line with good nodulation in breeding for enhanced N<sub>2</sub>-fixation (Rosas & Bliss, 1986) was found to be among the poorest nodulators when many genotypes of *Phaseolus vulgaris* were compared in Queensland, Australia (Redden et al., 1990).

Many workers observed large variations in the ability to nodulate and fix nitrogen among groundnut cultivars. Non-nodulating plants were observed in 13 crosses. Genetic analysis indicated the involvement of two independent recessive genes for nodulation. Some progeny of these crosses also formed big nodules, the genes for this trait seemed to be controlled by host plant. Adu and Misari (1989) found in case of groundnut that inoculation with *Rhizobium* strain NC-92 on Cvs Robut 33-1, P452-4 and RRB was most effective in improving nodulation, pod yield and shelling percentage but increased N<sub>2</sub> concentration of plant. Sridar et al. (1989) found that in groundnut, seed inoculation with *Rhizobium* strains increased nodulation and pod yield. Inoculation with strain TNAU 14 was most effective in increasing yield in Cv VR 1.1 and CO.2 than the control. Cv JL 24 gave the highest yield with strain NC92.

Zhang et al. (1988) found in pot trials with soybean cv Hefeng 26, 28 and 29 inoculated with each of five strains of *R. japonicum*. Out of five strains, strain 2053, 2057, 2058 were most effective in increasing the yield than US commercial strain 61A67. In all the three cultivars, the number of nodules was increased by 17.06 to 31.20% when inoculated with strains 2053, 2057, 2058 while with 61A67 the number of nodules however, decreased in Hefeng 28. Baliko et al. (1989) reported that inoculation had no effect on yield but increased the protein percentage in soybeans. It is also reported that use of cultivar specific inoculation would promote the multiplication of the most efficient strains. Champion et al.

(1992) studied that effect of mixed inoculation of an efficient and an inefficient variant of *Bradyrhizobium japonicum* on symbiotic performance in soybean. Lee soybean was initially inoculated with either an efficient or an inefficient USDA 110 colony morphology variant. The opposite variant was then applied after 0, 2, 4, 8 or 13 days. Delayed inoculation with the efficient variant resulted in the progressively decreased symbiotic performance. In a subsequent competition experiment, soybean cultivars Lee and Ramson were inoculated with efficient and inefficient variants at ratios of 1:1, 1:10 and 10:1. More nodules were formed by the efficient variant than were expected by chance. Significantly reduced dry weight and whole plant nitrogen contents were noted when the inefficient nodules was present in more than 50% of the nodules.

Sikinyi and Kimani (1988) when inoculated 12 genotypes of pigeon pea with two *Rhizobium* strains indicated that the response to a given *Rhizobium* strain was genotype specific. Inoculation increased nodulation and grain yield and reduced days to flowering.

Kremer and Peterson (1983) found that cowpea and peanut plants inoculated with selected *Rhizobium* strains produced more dry matter, contained more total N<sub>2</sub>, and produced higher seed yields than inoculated plants.

Leghaemoglobin (Lb) is a heme containing protein which binds with O<sub>2</sub>. This protein is produced in nodules in symbiotic association of both partners. Chopra and Subba Rao (1967) found positive correlation between and plant nitrogen content in *Trifolium alexandrinum* and *Cicer arietinum* at different stages of plant growth. Sidhu et al. (1967) also found a direct correlation between the Lb quantity and the total plant nitrogen in six legumes i.e. *Sesbania aculeate*, *S. macrocarpa*, *S. serica*, *Crotalaria juncea*, *Vigna sinesis* and *Cyamopsis tetragonoloba* grown under pot house conditions with or without phosphorous. Dadarwal et al. (1982) determined leghaemoglobin contents in two effective green gram *Rhizobium* strains 8-24

(slow growing and Hup<sup>+</sup>) and M11 (fast growing and Hup<sup>-</sup>) in six hosts of cowpea miscellany. All the host nodules formed by M11 contained 1.5 to 2.0 times more Lb than nodules formed by the other strain S-24. Effective functioning of strain S-24 at low levels of leghaemoglobin suggested the involvement of hydrogen recycling enzyme in maintaining appropriate oxidation-reduction potential in nodules. Subsequently, isogenic Hup<sup>+</sup> derivatives of strain S-24 were found to contain more amount of leghaemoglobin, whereas isogenic Hup<sup>+</sup> derivatives of M11 had lower level of leghaemoglobin in three host species confirming their earlier observations (Dadarwal & Garg, 1988).

Kremer and Peterson (1983) found that increased grain legume production depends upon effective symbiotic di-nitrogen fixation through successful legume inoculation. Inoculants containing high numbers ( $\geq 10^7/g$ ) of effective *Rhizobium* must withstand adverse field conditions. Field studies were performed to determine the effects of selective *Rhizobia* in two different inoculant carriers on inoculation and performance of three grain legumes. The experiments indicate that an improved inoculant provided high numbers of effective *Rhizobium* at planting. Through subsequent effective nodulation, oil based inoculants yield and nitrogen fixation by legumes due to increased nodulation by the superior N<sub>2</sub>-fixing strains of *Rhizobium*. Goyal and Ahmed (1988) preferred native *Rhizobium* strains over exotics for seed inoculation to get better crop stand and higher yields in various leguminous crops.

Nirmal and Singh (2010) conducted a trial during Rabi season of 2000-2001, 2001-2002 and 2002-2003 to study the effect of biofertilizers on nodulation of some genotypes of garden pea. The nodule number and weight per plant, leghaemoglobin content and nitrogenase activity increased linearly from active growth stage, reached to peak at flowering and then decline towards maturity irrespective of genotypes or microbial association in the rhizosphere.

Bhatt et al. (2013) reported that among seed inoculation treatments, dual inoculation of *Rhizobium* and PSB produced significantly higher seed and yield than inoculation with *Rhizobium*, PSB and control in case of Pea.

Stephen et al. (2019) investigated the host specific competitiveness to form nodules in *Rhizobium leguminosarum* symbiovar viciae (Rlv). The Fabae legumes such as Pea and Faba Bean form symbiotic nodules with diversity of soil Rlv bacteria. The variations of bacterial Pea explained differential abundance of Rlv genotypes in the nodules of Pea and Faba Bean.

Gisele et al. (2007) observed compatibility of *Rhizobium* genotypes within natural populations of *Rhizobium leguminosarum* biovar viciae for nodulation of host legumes. Populations of *Rhizobium leguminosarum* biovar viciae were sampled from two bulk rhizosphere soils and nodules of host legumes of Pea and Faba Bean were grown in the same soils to check their compatibility.

#### SOIL ECOLOGICAL FACTORS-NITROGEN FIXATION

The success of introduced *Rhizobia* on basis of nitrogen-fixation and crop yield depends upon their survival in soil and their ability to compete with native *Rhizobia* for nodulation with specific host cultivar. It has been found that infection of roots with *Rhizobium* and subsequent initiation of nodules and their functioning are processes that are sensitive to soil physical environment (Pate, 1976). A large number of soil ecological factors such as altitude, soil texture, soil organic matter, rainfall, temperature, available nitrogen, salt concentration etc. affect survival and competitiveness of *Rhizobia*. Definite relationship between soil taxonomic units and the crop yields for alluvial soils in Nawanshehar Tehsil in Punjab has also been reported by Satyanayaran and Dhir (1968). The other major considerations affecting either microbe, the host or their symbiotic interaction include soil acidity (Munns, 1977), flooding (Ladha et al., 1992), drought (Zaboltowicz et al., 1981) and oxygen concentration

(Bergersen, 1971). Rhizobium population is affected by altitude, soil type, different sites in a field and at different depths of the soil (Sharma & Purohit, 1978; Sidhu, et al., 1980; Pandher, et al., 1984; & Rupela, et al., 1987).

Virginie et al. (2007) investigated the effect of genetic variability of root and nodule establishment on N acquisition and seed protein yield. Legume nitrogen is derived from two different sources, symbiotically fixed atmospheric N<sub>2</sub> and soil N. These effects were investigated under field conditions in Pea (*Pisum sativum* (L.)).

### SOIL TEXTURE

Studies in the survival of root nodule bacteria in dry soil exposed to high temperature revealed higher survival rate in heavy textured soil and that clay in soil gives greater protection for the survival of Rhizobia (Marshall, 1964). Similarly, Marshall and Robertie (1963) reported that addition of clay soil to sandy soil checked the fall in *R. trifolii* population. Illite did not increase the survival and kaolinite reduced the number of survivors of three strains. Whereas the presence of montmorillonite increased the survival of two Rhizobium strains undergoing desiccation on fine sand. Further, the excess of clay content in soil was also reported to be less beneficial for the survival of *R. meliloti* (Chao & Alexander, 1982). They also reported that number of *R. meliloti* decreased as clay content and water at 15 bar suction increased. Mahler and Wollum II (1981) reported that *R. japonicum* and *R. leguminosarum* population were lowest in sand and clay loam soils and highest in sandy loam and sandy clay loam soils. Moawad et al. (1991) conducted a field experiment to assess the response to inoculation with Rhizobia in a clay loam soil of the Nile delta using Faba Bean (*Vicia faba*) for two successive winter seasons (1985 & 1986). Three selected strains of *R. leguminosarum*, TAL 634, NRC 65 and TAL 1400 were used singly or in combination with peat-based inocula in 1985 winter season. A significant seed yield response was obtained only with strain TAL 1400 in the 1985 season. In 1986 season, no significant yield response was obtained with any of the strains. Nutman

and Ross (1970) found lower population of Rhizobia in virgin soils continuously cropped with non-legumes than legumes. Rhizobial strains may behave differently in same soil. The variation in number of Cowpea Rhizobia was found to vary with soil and strain as reported by Afiana and Alexander (1982b). Materon and Hagedorn (1982) reported the occurrence of strains RP113-7, 162BBI LM1 and 162P17 in 94% of the assayed nodules whereas TAL was found in 83.8% of the nodules in vertisol soils. However, in an ultisol soil significant differences were detected. Similarly, Thamil Selvi and Rangarajan (1986) also reported 84% nodules in red soil and 75% in black soil in green gram with one strain and 60 and 80% of the nodules in two soils with another strain.

There are two opinions that agricultural production starts with soils under a given agro-climatic zone and the differences in soil and their ability to produce crop have been recognised since ancient times. The productivity ratings of different soils under defined management systems have been considered rewarding (FAO, 1976).

Helliwell et al. (2019) conducted an experiment to observe the role of plant species and soil condition in the development of rhizosphere part. They concluded that roots naturally exert axial and radial pressures during growth, which greatly affects the structural arrangement of soil at the root–soil interface. At some distance away from the root, densification of the soil was recorded dependent on soil texture and plant type. There was a significant soil texture × bulk density × plant species interaction suggested that pea and wheat grew better in the clay soil when at a high bulk density, compared with tomato, which preferred lower bulk density soils.

### ORGANIC MATTER

Vaughan and Ord (1981) found that <sup>14</sup>C-labelled Soil Organic Matter (SOM) is taken by Pea roots under axenic conditions. After 18h of culture, more fulvic acid or water-boile = d-soluble soil are taken up by the tissues than humic acid. The uptake is due to SO being adsorbed onto the root surfaces and cell

walls and to a process dependent on metabolism probably protein synthesis. Soil organic matter usually do not affect adversely the symbiotic process. Different kinds of organic matter have been used to see their effect on the symbiotic process. In early studies in 1955 increased yield of barseem fodder and seed were obtained by addition of farm yard manure and single super phosphate (Sen & Bains, 1955). Similarly, increased nodulation, leghaemoglobin content of nodules and N<sub>2</sub> fixation by the addition of FYM in Soybean (Dev & Tilak, 1976), increased seed yield in Chickpea and Groundnut (Kadam & Desai, 1983; Mishra et al., 1984) were observed. Addition of humic acid (Bhardwaj & Gaur, 1968) or humus (Mathur & Gaur, 1977) had opposite effect on N<sub>2</sub> fixation. Also addition of wheat straw in different legumes helped in better nodulation (Iswaran & Sen, 1970), nitrogenase activity (Shivashanker, et al., 1976b; Shivashanker, 1977; Shivashanker & Vilassak, 1978; Gaur & Mukerjee, 1979; & Sekhon et al., 1984). Other organic materials tested include gum and organic matter extract (Gaur & Bhardwaj, 1971), algal manure (Gangawane & Datar, 1979), charcoal peat and coal (Iswaran & Jauhri, 1980), cytozyme (Khurana & Dudeja, 1981), phosphocompost (Mishra et al., 1984), organic additives increased N<sub>2</sub> fixation of various crops tested except in case of cytozyme where no positive response to nodulation or gain in yield was observed.

Ramia et al. (2013) studied the effects of organic fertilizer on yield of pea, uptake of nutrient, microbial root colonization and soil microbial biomass indices in organic farming systems. They observed that application of C rich manure and compost failed to stimulate productivity of Pea.

#### TEMPERATURE

In *Pisum sativum* cv. Iran, roots are resistant to most *Rhizobium* strains at 20°C, yet this temperature is optimum for nitrogen fixation of already nodulated plants where nodulation proceeds normally at 26°C (Pate, 1977). The soil temperature affects root exudation, growth and survival of *Rhizobia*, root hair formation

in plants and the infection process by the microsymbiont (Ranga Rao, 1977). High temperature can prevent nodulation, or if nodulation does occur, can inhibit the activity of N<sub>2</sub> fixation in legumes (Day et al., 1978) even though the root nodules will be insulated from the highest temperature by the soil. Conversely, cool temperature lead to delayed development of plant, including delays in the formation of nodules, and so decreased rates of N<sub>2</sub> fixation. The optimum temperature for growth and N<sub>2</sub> fixation vary widely between legume species and reflect their environmental adaptation.

Differences in environmental adaption to high temperatures have been demonstrated between *Rhizobia* isolated from different climatic zones. More than 90% of Cowpea *Rhizobia* isolates from soils of the hot, dry Sahelian Savanna in Niger were able to grow on media at 40°C whilst few of the isolates from cooler humid regions could grow at this temperature (Eaglesham & Ayanaba, 1984). Only the high temperature tolerant isolates retained, or increased, their effectiveness in N<sub>2</sub> fixation in symbiosis with cowpea when the day temperatures were kept above 40°C.

Beena and Jayaram (2010) investigated the effect of temperature on seed water content and viability of green Pea (*Pisum sativum* (L.)) and Soybean (*Glycine max* (L.) Merr.) seeds. The study revealed that the seeds could withstand high temperature up to 70°C for 10 days continuously but high temperature reduced the rate of germination percentage and moisture content as well as seed and seedling vigour. This effect was observed in seeds of Pea resulted in appearance of hard seeds but not observed in Soybean seeds due to oil-rich nature.

#### SALINITY

Wilson (1970) found that nitrogen fixation in nodulated *Glycine* virtually stops in the presence of 74 or 148 mM Sodium chloride but the nodules rapidly recommence fixation when the salt treatment is ended. Salt accumulation in the nodules has been found to be limited, suggesting that reduced nitrogen fixation during salinity is associated with the



salt effects of the host legumes (Edwards, 1977). Salinity adversely affected the nodule number, nodule mass and nitrogenase activity in Chickpea (Balasubramanian & Sinha, 1976), cluster bean and moth bean (Garg, et al., 1984). In case of Pea increase in salinity there was a decrease in leghaemoglobin, total N, carbohydrate, amino acids and protein contents of the nodules and total N contents of plants (Siddiqui, et al., 1985). In Cluster Bean decrease in nitrogenase activity, GS and GDH activity under salt stress was attributed to the decrease in photosynthetic and respiratory activity of the plants. Saline tolerant strains of lentil were found to be more effective than saline sensitive strains (Rai, 1983). In Berseem nodulation was found to be normal up to 7-8 ECe, but only 58.2% of the nodules were effective as compared to the normal soils (Bajpai, et al., 1974). Senji and Lucerne were able to nodulate up to 12ECe. However with increase in ECe there was decrease in N-uptake, nodule number and mass (Batra & Ghai, 1988). Sesbania was able to grow up to ECe 11 and pH 10, without any detrimental effect on N<sub>2</sub> fixation (Rao, et al., 1987). Increased salinity and alkalinity led to decreased nodulation in Lucerne and Pea (Kumar & Garg, 1980). In Lucerne decrease in nodulation was due to reduction in root hairs, mucilaginous layer and infection thread formation (Lakshmi Kumari, et al., 1974). Alkaline conditions were more detrimental for N<sub>2</sub>-fixation than the saline conditions in Sesbania (Bhardwaj, 1974). Generally Rhizobia belonging to *R. meliloti*, *R. leguminosarum* and *R. trifolii* group showed optimum growth under saline-alkaline conditions, but host was susceptible these conditions (Bhardwaj, 1975). Saline-alkaline tolerant strains were found to be more effective in Sesbania (Lakshmi Kumari, et al., 1974). The process of root hair infection of legumes is particularly to saline stress, perhaps due to common cessation of root hair growth in these conditions. It may also be caused by the bacterial partner however as different strains of Rhizobia were found to show marked differences in the ability to infect and form nodules on Pigeon Pea under saline

conditions (Subba Rao, et al., 1990b). There was no reduction in nodule formation by *Alyosia platycarpa*, a wild relative of Pigeon Pea, at salinities (Sodium and Calcium chlorides) up to 8 dsm<sup>-1</sup> and effective nodules were even formed at 12 dsm<sup>-1</sup>, whilst number of nodules on Pigeon Pea were reduced at 4 dsm<sup>-1</sup> (Subba Rao, et al., 1990b). The Pigeon Pea following initial nodulation, grow in saline treatments (up to 8 dsm<sup>-1</sup>) with no harmful effects on the subsequent development or functioning of nodules (Subba Rao, et al., 1990a). Genotypes of Soybean vary in their sensitivity of growth and N<sub>2</sub>-fixation to salinity and this appears to be due to variation in the ability to avoid uptake of Cl<sup>-</sup> ions (Ranga Rao, et al., 1990).

Borucki and Sujkowska (2008) studied the effects of salinity of sodium chloride (NaCl) salt upon growth, nodulation, and root nodule structure of Pea (*Pisum sativum* (L.)) plants. In the experiment, the strains of *Rhizobium leguminosarum* bv. *viciae* were tested in nitrogen-free medium supplemented with 0mM, 25mM, 50mM and 75mM concentrations of NaCl. The results were indicated that 25mM concentration of NaCl stimulates the nodule formation, but treatment with 50mM and 75mM NaCl reduced the growth of plant, nodulation and nodule number.

#### pH OF SOIL

Thornton and Davey (1983) tested 33 strains of *R. trifolii* for the effects of soil acidity on nodulation, nitrogen fixation and dry matter production with “Mt. Barker” Clover (*Trifolium subterraneum* (L.)). Clover plants were grown in Norfolk loamy sand adjusted to 7, 34 and 57% Al saturation at acidity levels of pH 5.9, 5.1 and 4.4, respectively. Results from this experiment show that *R. trifolii* exhibits a large and varied tolerance to soil acidity in symbiotic association with host. Laboratory screening of *R. trifolii* in liquid medium, based on ability of *Rhizobia* to grow in acid media (pH 4.2) containing Al (15µM) identified 54% of the strains that proved symbiotically acid sensitive. Over 70% of the strains pre-screened as acid tolerant ranked in the top half of all the strains in total N accumulation in most acid

soil treatment. Aluminium toxicity occurs in acid soils affecting nitrogen fixation in nodulated legumes (Munns, 1977). The more important and common limiting factors in soil for N<sub>2</sub>-fixation in developing countries of tropics are soil acidity and toxic levels of exchangeable Al and Mn. The effects of acidic soils on nitrogen fixation has been studied in lentil by Rai and Prasad (1983) and in soybean by Barthakur and Sarmali (1984). Lentil inoculated with normal *Rhizobium* strain or its acid tolerant mutant was grown on acid soils. Acid tolerant mutant produced greater number and mass of nodules, fixed more N<sub>2</sub> than the parent strain in low pH soils. Application of lime and potash was found to be essential for boosting up soybean production in acidic sandy loam soils.

Bukvic et al. (2007) was conducted an experiment to evaluate the effects of seed age and water solution pH on field pea (*Pisum sativum* (L.)) germination energy, germination, seedling root length and stem height. They were tested seeds aged 33 and 21 months old and four water solution pH levels (5, 6, 7 and 8). The value of all the traits recorded was significantly higher for 21 months old seed. Different water solution pH exerted an influence on germination, seedling root length, stem height and germination energy. The germination energy was found to be significantly higher at pH=7 whereas germination and root length was highest at pH=6. Furthermore, stem height of seedlings was highest at pH=5.

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