REVIEW OF LITERATURE
CHAPTER - II

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The survey conducted on Rhizobium under Department of Biotechnology, New Delhi, sponsored project at Department of Soil Science, IGKV, Raipur showed that more than 50 per cent cultivated area of Raipur, Durg and Rajnandgaon, districts of Chhattisgarh region do not have native Rhizobium of different leguminous crops (Katre et al., 1997).

Hence, it is essential to identify the effective rhizobial stains for different legumes grown in Chhattisgarh region, so that suitable one's on mass multiplication can be released to farmers for increasing legume productivity per unit area of this region.

The work pertaining to different aspect of Rhizobium-legume symbiosis and other work relevant to the present investigation has been covered in the review that follows:

2.1. Temperature and Rhizobium-legume symbiosis:

The crop nodulates very poorly, especially in Chhattisgarh region. Poor nodulation of groundnut and soybean can probably be ascribed to the high temperature (nearly 50°C in upper 5.0 cm soil layer) prevailing in this region during planting season of the crop. Adverse effects of temperature on nodule formation and development have been very well documented in clover...
(Pankhurst and Gibson, 1973); Pea (Frings, 1976); Lotus and *Stylosanthes* (Rao, 1977) and soybean (Munevar and Wollum, 1981).

Soil surface temperature range of Chhattisgarh region crosses to about 60°C (air temperature reached upto 48°C and air humidity drops upto 3 to 4 per cent) during summer season, destroying rhizobial population. Surface soils almost become sterile. This condition results in poor nodule formation in legumes grown under such situations resulting in very low BNF. This is one of the major causes of low legumes, productivity in this region (Gupta 1995b; Anonymous 1996).

*Laxminarayana et al.* (1989) reported that the high temperature in semi-arid zones could be responsible for the low nodulation of pigeon pea because the plasmid carrying the nodulation genes is cured at 40-45°C giving rise to non nodulating mutants.

There have been a number of investigations on the effect of root temperature on nodulation and infection process of temperate species of clovers (*Trifolium spp.*) on agar slopes in environment controlled growth chambers (Gibson, 1963, 1965, 1966 and 1967). The results point out that below 10°C, root-hair infection by *Rhizobium* is retarded, whereas at 24°C and above, the rate of infection is enhanced. The most congenial range of temperature for bacteroid tissue formation in nodules appears to be 20-30°C, although nitrogen fixation remains unaltered in the range of 12-32°C. However, these results are dependent on variations between *Rhizobium* strains and host cultivars.
The effect of day temperature on root nodulation of soybean (Glycine max) and chickpea (Cicer arietinum) has been studied in pot trials in environment controlled growth cabinets (Dart et al., 1975). In soybean, one of the strains of Rhizobium was most effective at 33°C, while others showed no difference in effectiveness at 21°C. In chickpea, none of the bacterial strains produced nodules at 21°C and at root temperatures beyond 32°C.

Rhizobia are known to survive in stored dry soils for several years (Sen and Sen, 1958). An isolate of Rhizobium sp. (cowpea type) from 38 year old stored Andaman soils could tolerate 45°C and produced nodules on roots of Vigna mungo in experiments using Gibsons-tube method for testing nodulation under aseptic conditions. Further testing under field conditions revealed that this heat tolerant strain of Rhizobium significantly increased grain yields of Vigna mungo (Subba Rao et al., 1993).

Gibson (1971) found that the maximum temperature for nodulation varied with Phaseolus atropureus and tylosanthes humilis and had ability to nodulate more readily at 36°C than the other species examined. Further, Nakul et al., (1993) observed that the strains were tolerant to intermittent heat treatment at 45°C for 10 days hence, were chosen for temperature tolerance. Temperature is a critical factor in the infection and nodulation in legumes. The process of infection, nodule development and nitrogen fixation requires different maximum, optimum and minimum temperature. It may vary from species to species (Gibson, 1977). Dart et al. (1976) found evidence of difference in
*Rhizobium* strains in adapting to different temperatures. The optimum temperature for nodulation of legumes is about 27-30°C for tropical areas.

Our country comes under tropical zone where the temperature sometimes shoots up very high. So the viability of *Rhizobium* is greatly reduced. A favourable temperature for multiplication of most species of *Rhizobium* is up to 40°C as reported by Bhriguvanshi and Gangwar (1984). Day *et al.* (1978) and Kritovich *et al.* (1981) reported that bacterial growth is optimum at 37 to 42°C after which there is sharp decline. Similarly, Singh and Khurana (1992) have clearly mentioned that *Rhizobium* cells tended to loose their nodule inducing ability when inoculated at elevated temperatures. Moisture stress further added to the decline in survival and in the proportion of Nod+ cells. Pareek *et al.* (1990) have also reported that legume-*Rhizobium* symbiosis is highly affected by soil moisture and temperature in Tarai belt of U.P.

### 2.2. Importance of strain selection:

The first and most important step in bio-fertilizer production is the selection of efficient strain. This involves careful experimentation in the laboratory and field, which is possible only in well-equipped institutions with trained experts. Presently, bio-fertilizer strains are selected by i) extensive screening ii) mutagenesis and iii) genetic engineering methods. However, most of the bio-fertilizer strains used in India have been obtained through screening techniques while a few strains have been selected by mutation (Balasubramanian, 1992; Tilak, 1991; Siddaramaiah & Bagyaraj, 1981 and Kumar & Shrivastava, 1994). The only strain of *Azospirillum* developed by the
Tamil Nadu Agricultural University Scientists through the modern genetic engineering techniques is yet to be released for mass production. The performance of the selected strains have to be tested in field and hence takes nearly 3 to 5 years for releasing an efficient bio-fertilizer strain for field application (Balasubramanian, 1992).

Even though, we have national strains identified as efficient, there is still scope for improving the efficiency or for identifying a location specific better one (Bergersen, 1970; Kumar & Srivastava, 1994 and Halliday, 1984) One has to search continuously for more efficient strains. This can be accomplished by isolating and screening numerous wild type strains or by genetic manipulation and screening numerous mutants. Since the former method is time consuming, we are trying the mutagenesis approach for strain improvement. Initially the inoculant strains are selected for good performance under field conditions. Such strains are subjected to mutagenesis using chemical mutagens and transposon element (Palaniappan, 1992).

Strain efficiency reflects the ability for survival and multiplication in the carrier and soil, growth rate, tolerance to environmental stress, symbiotic properties such as nitrogen fixation, growth stimulant production etc. and competition with native flora existing in soil (Tilak, 1991; Palaniappan, 1992 and Subba Rao et al, 1993). In the case of Rhizobium inoculants, the presence of native (soil) rhizobia poses a problem in the form of competition. Since the native rhizobia are well adapted to the soil conditions, they are more competitive and are able to occupy more nodules of the host plant. Often the
nodules formed by native rhizobia are low in nitrogen fixing efficiency, which necessitates evaluating inoculant strains for competitiveness. The competitiveness of inoculant strains is tested by a serological method called Dot Immuno Blot Assay (DIBA). By this method we are able to quantify the number of nodules formed by inoculant strain (Palaniappan 1992). Legumes are well known to improve soil fertility through their ability to fix atmospheric nitrogen. Several workers have studied the possibility of inoculating the seeds of legumes with effective strains of rhizobia in order to increase yield of legumes (Sundara Rao, 1974; Habish and Ishaq, 1974). In Karnataka too rhizobia have been isolated, screened for their symbiotic response under different soil and agro-climatic conditions, and efficient strains have been developed for groundnut, redgram, greengram, blackgram, bengalgram and peas.

Quilt (1975) observed considerable variation in the performance of \textit{Rhizobium} strains when tested against different varieties of pigeon pea. Symbiotic variation of \textit{Rhizobium} with pigeon pea in relation to dry matter yield, N percentage of the crop and amount of N fixed has also been observed by Ramaswami and Nair (1965). Variety strain specificity in pigeon pea has been observed by Khurana \textit{et al.} (1978). Singh \textit{et al.} (1979) and Khurana and Phutala (1980). They have reported that nitrogen-fixing potential of \textit{Rhizobium} strain depends much upon its compatibility with the variety or genotype of the host.

Rhizobia were isolated from root nodules of horsegram grown in different parts of Karnataka. Morphological, physiological and nodulation tests were made to
confirm their identity. Twenty isolates of rhizobia thus obtained were screened for their symbiotic response with horsegram grown at Bangalore in microplots. Five isolates proved promising for horsegram. These five isolates were studied for their performance in different soils and agro-climatic conditions of Karnataka by taking up a multi-locational field trial at 4 different horsegram growing areas. Strain UASB 87 was found to be uniformly effective in all the places (Siddaramaiah, 1981).

Roots of black locust (*Robinia pseudoacacia*) was examined by inoculating seedling root zones with samples of soil collected from the USA, Canada and China. Bacteria were isolated from nodules, sub-cultured and verified to be rhizobia. The 186 isolates varied significantly in their resistance to antibiotics. Most isolates showed intermediate antibiotic resistance. Nitrogen fixation, total nitrogen accumulation and plant growth varied significantly among black locust seedlings inoculated with the representative isolates. It is concluded that great variation exists among *Rhizobium* spp. that nodulate black locust, and selection of strains for efficiency of the symbiotic association is possible (Batzli et al., 1992).

The use of efficient strains of *Rhizobium* as seed inoculant has proved to be the cheapest and most effective way to increase the yield of pulse crops (Kumar and Shrivastava, 1994). According to them the efficiency of local isolates/strains showed better performance (GKP3, GKP3 and GKP5) and was comparable with those of recommended ones (RCR 3824, VR 169, VR 441 and
VR 420). The nitrogen fixing efficiency of *Rhizobium* is highly influenced by the agro-climatic conditions prevailing at a particular place.

The prime objective of legume inoculant manufacturer is to provide highly efficient *Rhizobium* culture. Attempts have been made by several workers to correlate efficiency with (i) cultural and physiological characteristics (ii) antigen structure (iii) mutations (iv) phage sensitivity and (v) sensitivity towards antibiotic action (Jordan, 1962; Graham, 1963; Schwinghamer, 1964; Date, 1965). But, the most ideal way of determining efficiency is to conduct growth tests in leonard jars, pot culture and field evaluation. With the knowledge of cross inoculation group, it has become easier to select the efficient strains and has also been possible to produce composite culture.

The nitrogen fixing ability of a rhizobial strain can be determined by growing plants in sand cultures devoid of nitrogen source. Under these circumstances dry matter and protein contents of plants are directly related to the ability of rhizobia to provide nitrogen. However, several workers observed that strains showing equal efficiency under the laboratory conditions may show marked differences in ability to nodulate under field conditions. Burton (1976) suggested the following reasons for it:

1. Differences of strains related to soil fertility level and pH of the soil.

2. Inherent variations in rhizobial efficiency that come in the view point under the nitrogen stress provided by the plant.
3. Variations in the ability of the nodulating bacteria to resist the antagonistic microflora.

4. Differences in competitive qualities of the rhizobial strains.

The effectiveness or ineffectiveness of strains of *Rhizobium* cannot be determined by laboratory cultural tests. Nodulation tests with appropriate hosts can only provide clear-cut evidence. Ineffective strains of rhizobia form ineffective nodules which are generally small and contain poorly developed bacteroid tissue showing accumulation of starch in host cells which do not contain *Rhizobium*. The bacteroids of ineffective nodule contain glycogen. On the other hand, effective nodules formed by effective strains are well developed, pink in colour due to leghaemoglobin and the bacteroid tissue is well organized with plenty of bacteroids (Bergersen and Brigss, 1958; Jordan, 1962; Dart and Mercer, 1963).

The amount of N₂ fixed by a leguminous crop has been variously estimated. Based on conventional methods of estimating the total amount of N₂ fixed by nodulated legumes, Nutman (1975) reported that substantial amounts of nitrogen is fixed by grain as well as forage crops in temperate and tropical climates. Based on the "A" value method of Fried and Broeshart (1975), estimates of accurate amounts of N₂ fixed under field conditions have been made in field experiments organized by International Atomic Energy Agency (IAEA), Vienna, in different parts of the world using labelled ammonium sulphate. Some of the results point to the importance of nodulated legumes in fixing considerable amounts of nitrogen from the atmosphere.
2.3. Method of inoculation and benefit:

Agar based cultures are the quickest way to inoculate plants in small experiments. The surface growth of *Rhizobium* on agar is scraped with needle or scalpel and suspended in water, which is used to sprinkle seeds before sowing.

Carrier based cultures are mixed with minimum amount of water to form a slurry with the addition of 10 per cent sugar or 40 per cent gum arabic and the slurry is sprinkled on seeds. The seeds are allowed to dry in shade and sown.

Rhizobia were applied to seed because this was an easy, convenient way to establish the bacteria in the root zone of the developing seedling. The laborious task of spreading tons of soil to provide a few rhizobia could thus be bypassed (Peppler and Perlman, 1979).

In the United States the normal rate of application is 4.4 gm of inoculum per kilogram of seed regardless of seed size. The amount of water is greatly reduced for the larger seeds. The big seeds naturally receive larger number of rhizobia per seed. According to Burton and Curley (1965), they need more rhizobia for effective nodulation. Nodulatory standards often specify a minimum of $10^3$ viable rhizobia per seed regardless of size. An inoculant with $10^6$ rhizobia per gram would meet this standard with peas (Peppler and Perlman, 1979).

In the USA and Australia, pelleted seeds are often used to establish legumes in acid soils or to avoid the hazards of pesticides or fertilizers (Burton, 1979; Brockwell, 1977). The usual method of pelleting involves additions of 40
per cent gum arabic or 5 per cent carboxy methyl cellulose to the inoculant slurry before application to seeds. Finely ground calcium carbonate capable to passing 300 mesh sieve is added to freshly inoculated wet seeds in a container and mixed rapidly for 2 minutes until the seeds are coated. Besides lime, other forms of pelleting agents such as dolomite, gypsum, bentonite, rock-phosphate, talc, charcoal and basic slag have been used to establish soybean in problem soil (Chhonkar et al., 1971).

Responses to legume inoculation have been demonstrated with major legumes such as pigeonpea (Cajanus cajan) and mungbean (Vigna mungo). The benefits to the farmer can be sizable by proper *Rhizobium* inoculation as shown for some of the important grain legume.

Besides, peat-based inoculants other forms used in the USA are granular soil inoculants where marble, calcite grains or cores are wetted by peat based cultures using adhesives. Such granular inoculants could be broadcast by air plane. Most of these improvements tend to be expensive and their possible use in developing countries is hence limited (Subba Rao et al., 1993).

2.4. **Role of Rhizobium and its effect on biomass and N-uptake**

Most of the soils contain rhizobia, which range in effectiveness from highly beneficial strains to those of little or no value. Studies with peas and other common beans indicate that the host plant is the dominant factor in assessing the effective and ineffective strains of rhizobia (Burton, 1952, 1964; Burton et al., 1954). All these tests show that some strains of rhizobia are more
acceptable than others to a particular host plant. But it is not clear, however, whether this property which results in nodulation should be attributed to the rhizobia, to the host plants or to both of the symbionts, because a rhizobial strain considered highly competitive on one host genotype might have the opposite rating on another genotype. Any competitiveness rating assigned to a rhizobial strain should be related to a defined rhizobial population as pointed out by Burton (1952). The plants nodulated by ineffective rhizobial strain showed lower dry matter yield, grain yield and nitrogen content irrespective of number of nodules than the effective strains of Rhizobium.

Groundnut plants fixed most of its nitrogen requirement when nodulated with effective nitrogen fixing bacteria (Peltiti et al., 1975). Inoculation with effective *Rhizobium* strains have increased pod yields in fields where groundnut had not been previously grown (Burton, 1976). Similarly, Sundara Rao (1971) obtained higher yields of groundnut with Rhizobium inoculation. According to Nambiar and Dart (1982) there is an increase in yield in the range of 2.8 to 40 per cent (60 to 1000 kg pods/ha) on rhizobial inoculation over the control. *Rhizobium* strains did not affect plant population number and weight of nodules, but significant differences were obtained in the pod yield (Anonymous, 1982). Similarly, Ramakrishnan (1985) reported that direct seed inoculation of groundnut with *Rhizobium* has not adversely affected the seed germination and plant standard. Subba Rao (1986) found that grain yield of legume was 50 per cent increased on *Rhizobium* inoculation over uninoculated control.
Alagawadi et al. (1993) found that inoculation of groundnut seeds with Rhizobium significantly increased yield over that of the uninoculated control. Inoculation increased nodule number, dry weight of nodules/plant and N content of plants at 120 days after sowing. An application of N to a plot with uninoculated plants reduced nodule number and dry weight compared with the uninoculated control. According to Joshi and Kulkarni (1984) that seed inoculation with Rhizobium strain increased the pod yields of groundnut when given 50 kg N/ha.

Poi and Kabi (1983) found that inoculation significantly increased fresh weight and N2 content of pot grown plants. Plants in adjacent fields had an average of 44 and 55 nodules/plant and recovery of introduced Rhizobium strains was poor due to the high competitive ability of native strains. Similarly, Wahhab and Bhuiya (1984) reported that oil and protein contents in groundnut were positively correlated with nodules/plant but not with nodule size. Kulkarni et al. (1984) found that when seeds of Arachis hypogaea varieties J-11 and Robut 33-1 were sown in soil inoculated with Rhizobium pod yield increases over those of uninoculated controls ranged from 9.8 per cent to 40.8 per cent. It also shows increase in nodules/plant and plant dry weight, as well as the highest increase in yield.

Pod yields consistently increased by inoculation. The increase was from 18 to 34 per cent in Hyderabad. Inoculation with the pure strain gave higher yields than a mixture with other strains of Rhizobium sp. (Nambiar et al., 1984b).
Nambiar et al. (1983) found that in greenhouse studies, the shoot and nodule weight/plant of groundnut increased with increase in inoculum density from $6.1 \times 10^2$ to $3.2 \times 10^9$ rhizobia/seed. In other experiment the number of nodules/plant increased from 6 to 11 at $2.7 \times 10^2$ to 143-243 at $2.7 \times 10^8$ rhizobia/seed but $N_2$ fixation at $10^6$ rhizobia/seed was close to that at $10^8$. Nambiar (1985) found that inoculating with sufficient numbers of an effective *Rhizobium* strain, applied as a liquid slurry below the seed, increases the yields of certain groundnut cultivars. Inoculation with *Rhizobium* strain increased the proportion of nodules from 25-32 per cent in the first season to 41-54 per cent in the second season.

Inoculation response in pigeon pea have been reported by Yadahalli and Jayaram, 1975; and Simhadri and Tilak, 1976 in terms of increased dry matter, nitrogen uptake and grain yield. While, Subba Rao, 1976, concluded from the 11 field experiments, conducted at different locations in India, the response of *Rhizobium* inoculation to pigeon pea (*Cajanus cajan*) was variable. At some locations there was no response to either *Rhizobium* or nitrogen application whereas at other locations, due to inoculation, an increase in the yield up to 68 per cent was recorded.

Wange and Patil (1996) reported that rhizobial strain performed significantly well in improving nodulation, plant height shoot dry weight, pod number and grain yield of tur (pigeon pea) crop in field conditions over nitrogen and uninoculated control. For their study they took 3 cultivars and 5 strains of *Rhizobium* and suggests that strain of pigeon pea rhizobia respond differently.
with different cultivars of same host species. Singh (1996) also mentioned that nodulation and grain yield of pigeon pea can be increased significantly due to seed inoculation with *Rhizobium*. Further, Devi and Gupta (1996) they observed that second inoculation of pigeon pea with *Rhizobium* increased plant growth with and without green manuring.

Raut and Ghonsikar (1980) reported that greater weight of nodules per plant, greater N$_2$ accumulation per hectare; number of nodules/plant and grain yield /ha. can be obtained by inoculation of *Rhizobium* on pigeon pea, while studying the response of two pigeon pea cultivars to inoculation with *Rhizobium* at Parbhani (MH) using Vertisol (pH 8.1). They are of opinion that here beneficial inoculation with *Rhizobium* can be to a pigeon pea crop when it is grown on Vertisol on which this crop had not previously been grown. They used two cultivars BDN-1 and C-11 and Rhizobium strain No.50, isolated locally and further concluded that BDN-1 produced significantly, greater weight of nodules per plant than C-11, which resulted in a significantly greater accumulation of N$_2$/ha. This was despite a lower but non-significant number of nodules/plant and grain yield /ha in BDN-1.

Tippannavar *et al.*, (1991) while conducting experiment on response of phospho-bacterium and *Rhizobium* inoculation to pigeon pea in northern dry zones of Karnataka found favourable response of inoculation to pigeon pea among the bio-fertilizers tested. The data indicated the percentage increase of average number of pods/plant, average number of nodules/plant and grain yield over uninoculated control ranged from 0.49 to 54.98, 2.75 to 52.75 and 0.07 to
20.07, respectively. The nodulation characters were found to be superior in *Rhizobium* treatments as compared to combination treatments.

Schroder (1989) reported that nodule number weight and total N content of pigeonpea can be increased by inoculation with superior *Rhizobium* strains. The inoculation with an effective rhizobial strain is capable of surviving and competing successfully with native rhizobial population for better legume yields (Sree Kumar, 1989). Manoharn *et al.* (1990) observed that number of nodule per plant was positively correlated with nodule mass per plant. Similarly, Zalawadia and Patel (1983) reported that the available P in surface soil was significantly co-related with dry matter, yield and P uptake at all the stages of crop growth. Sairam *et al.* (1989) mentioned that number of nodules and their dry weight increased linearly with the application of phosphorus at the growth stages.

Results have also been reported by Mc Neil *et al.* (1981) showing that up to 40 days stage there was a rapid increase in both nodule number and its dry weight on inoculation of the *Rhizobium*. Jarek (1989) also found that the greatest positive effect of green pea inoculation with *Rhizobium leguminosarum* strains in plant weight, while the strains had the lowest effect on plant height.

Seikhon *et al.* (1992) concluded that dual inoculation of the Hup + *Rhizobium* strain and VAM significantly increased nodulation, nitrogenase activity, plant nitrogen and phosphorus content and plant biomass compared to single inoculation of either organism and dual inoculation with Hup and VAM fungus.
Young et al. (1989) reported that inoculation with *Rhizobium* alone significantly increased nodulation, nodule weight and nitrogenase activity of nodules; and increased soybean yields up to 134 per cent. He also found that single or mixed inoculation of rhizobia with VAM can increase soybean yields and may replace N fertilizers in soybean/rice rotation. Baruah et al. (1995) also found that inoculation with *Rhizobium* alone stimulated nodulation and nitrogen fixation in green gram. Dual inoculation of *Rhizobium* with VAM further significantly increased legume growth, nodulation and nitrogen fixation.

Joshi et al. (1989) found that *Rhizobium* inoculation significantly increased the yield of groundnut by 14.4 per cent and of soybean by 10.69 per cent over no inoculation. They also suggested that the increase in yield was due to favourable effect of *Rhizobium* on pod number and test weight. Similar results were also reported by Muhammed et al. (1973) in groundnut. Jena (1990) estimated that N₂ fixed by groundnut at 60 DAS is equal to the N₂ fixed at harvest. Tiwari et al. (1989) observed that there was a marked variation in terms of nodulation and groundnut yield due to effectiveness resulting from application of different strain. Senaratne (1984) concluded that strain of *Rhizobium* for a particular legume can fix maximum amount of nitrogen only when inoculated at specific soil and environmental conditions.

Nayak et al. (1989) reported that inoculation of groundnut seeds with rhizobial culture significantly increased the number of nodules per plant, test weight of kernel and yield (35.01%) over control. Application of 20 kg nitrogen per hectare to the inoculated seeds further improved the number of nodules and
filled pods per plant. He also found that application of 20 kg nitrogen along with 40 kg P₂O₅ to the inoculation seeds increased the groundnut yield by 83.9 percent over control.

It has been an established fact that the bacterial inoculation of legumes is quite necessary for their proper establishment when grown in new place (Jenkins et al. 1954). Nodule bacteria (rhizobia), in association with leguminous hosts, fix at least 90x10⁶ metric tons of N annually in the world. This is more than twice the amount of N, used in chemical fertilizers and more than one-half the total amount of this element fixed biologically each year (Hardy and Holsten, 1972). Mahanta (1969) reported that rhizobia supply nitrogenous compounds to the host plant (leguminous plants). Rhizobium spp., have the ability to infect roots of leguminous plants, form nodules and work symbiotically with their host in fixing molecular N. The Rhizobium leguminous plant association offer the greatest promise of all systems for providing the nutritious protein food which will be needed in the years ahead (Peppler, 1979).

Simhadri and Tilak (1976) reported that increased nodulation and leghaemoglobin synthesis and seed yield of pigeon pea when inoculated with Rhizobium. Increased grain yield, which was approximately equivalent to that obtainable with 40 kg N/ha have been reported by Subba Rao and Tilak (1977). Increase in grain yield of pigeon pea by manipulating soil mineral N-level and using effective Rhizobium strains have been demonstrated by Quilt and Dalal (1979).
Bhargava et al. (1974) reported that inoculation with effective strains of *Rhizobium japonicum* was imperative for successful cultivation of soybean. There was better nodulation and higher bacteroid and leghaemoglobin content in the nodule in the case of inoculated plants.

Karle et al. (1980) observed similar type of result in nitrogen deficient soils. He also concluded that soybean yield is significantly increased with the use of specific Rhizobium culture. The dilution in inoculation decreases the yield of soybean. This gave conclusive evidence that nodulation is an essential attribute in production of soybean.

Rambalak (1964) obtained 10-40 per cent and 11-39 per cent increase in grain yield of soybean by inoculation of seeds with different strains (*Rhizobium japonicum*). Saxena and Mutsuure (1970) observed that soybean inoculated with bacterial culture and basal application of 20 kg N/ha produced as good or even better crop yield than application of 120 kg and 160 kg N/ha. Soybean responds spectacularly to *Rhizobium* application and grain yields are often increased up to 50 per cent over uninoculated controls since the soils were deficient in specific bacterium capable of nodulating soybean (Subba Rao, 1986). *Rhizobium* application to soybean increased the number of nodules/plant, plant height seed yield (from 0.90 to 1.28 tons/ha), number of pods/plant, oil yield and seed weight (Yazdi and Zali, 1978).

The weight of soybean plants was increased by 19-27 per cent, number of seeds per pod was increased by 11-18 per cent, yield was increased by 9 to 51 per cent and the numbers and weight of nodules increased by 19-30 per cent.
with seed inoculation (Sen and Palit, 1988). Maskey and Bhattarai (1981) observed that _Rhizobium_ inoculation increased the N content of roots, leaves and nodules of soybean cv. Bragg at the flowering stage.

Donwa and Quilt (1981) have reported increased dry weight, nodule weight and number and nitrogenase activity of 8 week old pigeon pea plant inoculated with _Rhizobium_. Application of P.K.S. + trace elements gave greater increase in shoot weight, nodule weight and number of nodule and N₂ase activity. Similarly, Khurana and Dudeja (1980) and Gupta (1980) reported that significant increase in nodulation, total plant nitrogen, total dry matter and grain yield of pigeon pea due to inoculation of _Rhizobium_.

Kim _et al._ (1989) found that _Rhizobium japonicum_ inoculation increased the number of nodules/plant. They also reported that N fixation was significantly increased by inoculation and seed yields were increased by 3-8 per cent. The increase in yield was due to favourable effect of _Rhizobium_ on pod number and test weight. _Rhizobium_ inoculation was more efficient source of N for field peas than mineral nitrogen according to Fluiczek (1978). Hulamani _et al._ (1972) reported that _Rhizobium_ and/or molybdenum treatment of pea seeds increased green pod yield during two seasons. Rao (1980) reported an increase in grain/seed yield resulting from the inoculation of legume (Bengal gram, soybean, arhar, cowpea, mung, urid and moth) seeds with _Rhizobium_ strains.

Lakshman Rao and Singh (1983) observed that _Rhizobium_ inoculation had marked influence on leghaemoglobin content of nodule tissue. They also concluded that the total leghaemoglobin content in the nodules is directly related
to the total nitrogen fixed by the chickpea. Singh and Choubey (1970) reported that proper inoculation with efficient strains of rhizobia is the cheap, easy and safe source of supplying nitrogen to legumes for boosting up their yields.


Tripathi and Edward (1980) also found that the culture inoculation to different leguminous crops invariably increased soil nitrogen over uninoculated control. Amongst various legumes tested, sunhemp and berseem recorded highest N enriching potentiality. Inoculation is so inexpensive that some farmers have taken to inoculating all pulses and fodder crops such as berseem and lucerne with *Rhizobium* as a routine practice to insure against crop failure (Subba Rao, 1986).

Prasad and Ram (1986) found that *Rhizobium* inoculation increased all the parameters over uninoculated control possibly owing to N₂ fixation and favourably affecting P solubilization in rhizosphere soil.

Rawat and Sanoria (1976 and 1978) studied the combined effect of *Rhizobium* and *Azotobacter* on bengal gram and found that the nodule weight as well as grain yield increased significantly over the uninoculated control and the increase in grain yield was 45 per cent. Rawat and Sanoria (1978) also reported that the grain in soil N ranged from 190 to 262 kg/ha. Dry weights of nodules and grain yield were also maximum due to combined effect of *Rhizobium* and *Azotobacter*. More-over, these variables were significantly correlated.
2.5. **Rhizobial population dynamics study:**

At flowering stage of crop growth, microbial population was increased considerably as compared to vegetative stage of crop due to higher root activities i.e. greater rhizosphere effect (Shetty and Rangaswami, 1969).

Louw and Webley (1959) reported that count of bacteria in the root region increased during the growing period of the plant. According to Nutman (1975) stimulation of the rhizobia is greatest at places where lateral roots emerge and generally extends to 10-20 mm from the root surface into the soil. Increased growth of *Rhizobium* in the rhizosphere is a response to excretion of energy source, amino acids, growth factors, especially B group vitamins, and enzymes by plant roots. The rhizosphere stimulation is a response to a complex mixture of substances. It was demonstrated by Rovira (1956), who was unable to replace fully the stimulating effect of root exudation by mixture of glucose, soil extract, amino acids and all growth factors known to be excreted by roots.

*Rhizobium* is pre-eminently a rhizosphere organism multiplying on the root surface and in the root surroundings, and within the mucilage layer of both legumes and non-legumes, but especially legumes (Rovira, 1961).

Podyapolskaya (1960) reported greater phosphate dissolving bacteria in rhizosphere of chickpea and mustard in comparison to non-rhizosphere soil.
2.6. Antibiotic resistance nature of Rhizobium strains:

Strain identification is a major problem in field experimentation with strains of Rhizobium. Where legumes are regularly grown, soils invariably contain some rhizobia capable of nodulating the particular host. It is, therefore, desirable to develop simple technique for identifying inoculant strains of Rhizobium spp. The use of genetic markers such as intrinsic levels of resistance to various antibiotics is one of the simple and rapid method for strain identification (Johnston and Beringer, 1975; Josey et al., 1979).

Microorganisms, in general, display an intrinsic resistance to the inhibitory or lethal effects of particular antibiotics. Such resistance may depend, e.g., on the absence or inaccessibility of those structural and/or functional features against which the antibiotic is effective. The antibiotic to which the resistance may be gained include aminoglycoside antibiotics (gentamycin, kanamycin, neomycin, paromomycin, streptomycin and spectomycin), chloramphenicol, erythromycin, penicillin, sulfonamides and tetracycline.

The intrinsic levels of resistance to host of antibiotics is developed by using different concentrations of antibiotics, and once the resistance pattern for a particular strain is developed it can be used to identify the strain (Saxena, 1998).

Subramaniam and Babu (1993) suggested that:

1. The Intrinsic Antibiotic Resistance (IAR) patterns are both strain specific and antibiotic specific.
2. The IAR technique is sensitive enough to discriminate between rhizobial isolates of the same legume host inhabiting different habitats.

3. The IAR patterns of the fast growing strains were dissimilar from those of slow growing strains.

Native *Rhizobium meliloti* from 50 sites in Mexico were grouped according to their sensitivity to 12 antibiotics into 10 strains. Efficiency of nitrogen fixation was found to vary with the host cultivars. The highest efficiency observed with the local cultivar “San Miguelito” (Olalde-Portugal and Pena-Cabriales, 1989). Similarly, Espiritu *et al.* (1988) tested rhizobial isolates of mungbean for their intrinsic resistance to two levels of six antibiotics and were evaluated for colony morphologies. They found that rhizobia consisted of equal proportions of isolates exhibiting ‘wet’ slimy colony morphology and those with small discrete ‘dry’ non-slimy colonies. The isolates were resistant to carbenicillin at 50 μg/ml and to neomycin at 5 μg/ml but showed variable responses to kanamycin, penicillin, rifampicin and streptomycin. The 111 isolates screened in this way diverged with 23 patterns of resistance. Cinani (1991) also tested 74 strains of *Rhizobium*, isolated from various legumes against a number of antibiotics. The strains produced 22 patterns of resistance. Thirteen strains showed a unique pattern of resistance and were considered as marked strains.

Roughley *et al.*, (1992) examined isolates of *Rhizobium* and *Bradyrhizobium* to determine the upper limit of their intrinsic resistance to both streptomycin and spectinomycin. He found that out of 38 isolates, 13 were
resistant to more than 600 ppm streptomycin and 29 isolates were resistant to
more than 1000 ppm of spectinomycin. He also found that there was no
relationship between extra-cellular polysaccharide production and resistance to
antibiotics. Vargas et al. (1992) found differences among strains of *Rhizobium
leguminosarum* bv *phaseoli* for tolerance to antibiotics. Tetracycline inhibited
growth of all rhizobia and 20 ppm of rifampicin, spectonomycin or genomycin
were found enough to inhibit growth of more than 93% of *Rhizobium* strains
tested. Ampicillin had the lowest inhibitory effect on rhizobia. Streptomycin
showed reduction in the number of strains able to grow, depending on the
concentration used. *Rhizobium* strains showed cross-resistance to antibiotics.
They also found that rhizobia from acid soils were less tolerant to antibiotics
than rhizobia isolated from non-acid soils. Hosny et al. (1991) observed that 60
per cent of the strains of *Rhizobium leguminosarum* under study were sensitive
to neomycin, kenamycin and streptomycin. Only 11.7 per cent could resist the
presence of all the three compounds at their highest concentrations.

Odee et al. (1997) reported that *Bradyrhizobium spp.* were more
sensitive to antibiotics (40 fg ml⁻¹) than *Rhizobium spp.*, contrary to the general
opinion which indicates that they are normally resistant. In laboratory tests,
growth of *Rhizobium spp.* was reduced by the 5 antibiotics tested (streptomycin,
paushamycin, agrimycin, spectinomycin and HPMTS (2-hydroxypropyl
methanethiosulfonate). However, agrimycin and HPMTS were only inhibitory at
1000 ppm (Patel et al., 1989).
Gupta et al. (1989) reported that the strains of *Rhizobium trifolii* which have different levels of intrinsic resistance to 8 antibiotic markers, caused significant increase in nodule DW, nitrogenous activity and green fodder yield of *Trifolium alexandrinum*. They also mentioned that nitrogenous activity was a reliable parameter for estimating yield. The competitive ability of different strains differed with different host cultivars. Intrinsic antibiotic resistance was not associated with loss of effectivity.

Three strains of *B. japonicum* that were resistant to streptomycin, spectinomycin and chlorate were isolated and field inoculation experiments were conducted with these strains on soybean in Japan. The chlorate resistant strains lacked nitrate reductase activity in anaerobic conditions. Antibiotic resistant and nitrate reductase deficient strains showed high symbiotic characteristics. Using a streptomycin resistant marker strain, inoculated *B. japonicum* was shown to mainly infect the taproot of soybean. IAR was observed in many rhizobial isolates from tropical and sub-tropical countries (Gamo et al., 1994).

2.7. Salt and temperature tolerance behavior of Rhizobium:

Saline soils are widespread in many parts of the world. In India, such soils prevail in the Indo-Gangetic basin in the north and are not suitable for raising food and fodder legumes. Salinity is caused by the accumulation of soluble salts of calcium, magnesium and sodium, mostly as chloride and sulphates. Application of large quantities of gypsum to the soil can ameliorate
these soils and render them fit for cultivation. Alternatively, pelleting leguminous seeds with gypsum can help in the establishment of legume crops.

Some fundamental studies carried out with lucerne plants (*Medicago sativa*) under bacteriological controlled conditions on agar slopes have revealed that *Rhizobium* can tolerate higher levels of all the above mentioned salts than the corresponding host plant (Subba Rao, 1986). According to Botsford and Lewis (1990) *Rhizobium meliloti*, like many other bacteria, accumulates high levels of glutamic acid when osmotically stressed. The effect was found to be proportional to the osmolarity of the growth medium. Sodium chloride elicited this response. They concluded that the excess glutamate made in response to osmotic stress by sodium chloride is derived from degradation of amino acids and transamination of 2-ketoglutamate.

Batzli *et al.* (1992) found that rhizobial isolates varied significantly in their resistance to sodium chloride. Hosny *et al.* (1991) in their salt and temperature tolerance studies found that 55 per cent rhizobial strains could only grow at a salt level of 1 per cent and only 2 per cent survived in 6 per cent sodium chloride. They observed no growth in 8 per cent sodium chloride. They also found that only eight strains could resist 45°C for 7 days incubation and fifty-nine strains (36.2%) could not tolerate temperatures up to 35°C.

The effective and efficient isolates of *Rhizobium* of *Sesbania bispinosa* were studied from the rice growing areas of Tamil Nadu, India. From 661 samples, *Rhizobium* was isolated and tested for nitrogenous activity. The most positive isolate (DMBR-001) was selected. This strain was studied in detail for
its tolerance to salinity and temperature tolerance. The observations showed that the strain grew well in salinity range of 0-600 millimhos (3 per cent of Sodium Chloride) and a temperature range of 28 to 44°C (Gopalkrishnan and Jeevanand, 1996).

Hungria and Franco (1993) found that two thermal shocks of rhizobial strains at the rate of 40°C per eight hours per day at following time markedly decreased nitrogenous activity and nodule relative efficiency of legume plants otherwise grown at 28°C.