CHAPTER - III
Chapter III

A. ISOLATION AND CULTURING OF Rhizobium STRAINS IN ARTIFICIAL MEDIA AND STUDIES OF THEIR PHYSICOCHEMICAL CHARACTERISTICS.

Rhizobium improves the fertility of soil by converting atmospheric nitrogen into nitrogen compounds for plants to synthesize proteins. It also converts organic substances into inorganic compounds in the soil, thus making them available as nutrients for plant growth. Rhizobium grows in pure culture and in reality, the normal population of a natural habitat comprises a very large number of different microbial species. The kind and diversity of microbial species growing in a region form characteristic of a natural environment. Cultural methods will reveal only these physiological and nutritional types compatible with the cultural environment.

Very often the microbiological analysis of soil is concerned with the isolation and identification of specific physiological types of organisms. For this purpose, the enrichment culture technique of Winogradsky and Beijerinck is most appropriate.

The microbial population in and around roots is considerably higher than that of root free soil; the differences are both quantitative and qualitative. It has been demonstrated that the microbiota of the rhizosphere is more active physiologically than that of non-rhizosphere soil. Holding (1960) reported that the average proportion of Gram-negative
bacteria in plant free soil was 7 percent whereas in the rhizosphere it was 20 percent. Plant roots affect microbial growth, and the plant in turn is affected by the increased activity of the microbial population of the rhizosphere. 

Rhizobia are soil saprophytes but their population are usually greater in plant rhizospheres than in the root free soil (Vincent, 1974). Legume rhizospheres generally contain more rhizobia than non-legume rhizospheres. There are examples of selective stimulation of rhizobia in rhizospheres of legumes capable of forming nodules with this rhizobia.

Symbiotic nitrogen fixation is accomplished by bacteria of the genus Rhizobium in association with legumes. Infection of the root system by the rhizobia bacteria is closely associated with the formation of an "infection thread" that develops in certain root hairs. The nitrogen fixing bacteria invade the host plant cells through this infection thread causing cell enlargement leading to the formation of abnormal growths (nodules) on the root system. The legume—rhizobium symbiosis is considered the most promising plant bacteria association for immediate increases in protein yield through biological nitrogen fixation. Cherry (1981) indicated that the ability of legumes to use atmospheric nitrogen was recognized a century ago, and in recent years there has been an upsurge of research into biological fixation, where leguminous plants play an unquestionable role in maintaining the
fertility of agricultural soils. Atwater (1885) confirmed the accumulation of nitrogen by leguminous crops. During the second half of the nineteenth century many Russian investigators also concluded that leguminous plants were of undoubted value.

Nodule bacteria are now divided to species according to their infective powers in relation to the host organism. Bergy's classification (1957) contains six species of \textit{rhizobium} i.e., \textit{Rh. leguminosarum}, \textit{Rh. phaseoli}, \textit{Rh. trifolii}, \textit{Rh. lupini}, \textit{Rh. japonicum} and \textit{Rh. meliloti}. In the 8th edition of Bergy's manual (Buchanan and Gibbons, 1974) the family Rhizobiaceae is divided into two genera, \textit{Rhizobium} and \textit{Agrobacterium}. \textit{Rhizobia} stimulate nodule production on roots of legumes and fix free nitrogen when in the symbiotic state within root nodules. \textit{Rhizobium} is divided into two groups, group-I has 2-6 peritrichous flagella and grow rapidly on yeast extract media and group-II has a polar or sub-polar flagellum and grows slowly on yeast extract media. The different species and races of nodule bacteria has certain differences in morphological and physiological properties. Some are efficient and of great value to the plant, the majority are only moderately effective in nitrogen fixation. The legume-inoculum relationship has been established and highly efficient nitrogen fixing strains of \textit{rhizobia} have been isolated for agricultural use.
Nodule bacteria of the various leguminous species grow at different rates in bacteriological nutrient media. Rapid growth is a feature of those from Clover, Pea, Bean, Black gram, Summer moong, Lucerne and Alfalfa; the slow developers come from Soybean, Lupin, Peanut, Gram and cowpea. On solid media, nodule bacteria usually form colourless, transparent mucilagenous colonies, of the type peculiar to "S" forms. Sometimes the rough colonies peculiar to "R" forms are found (Izrail'skii and Starygina, 1930).

The mucilage of the strains that develop slowly consists essentially of polysaccharides that are poorly soluble in water, whereas that of fast growing strains are composed of water soluble polysaccharides (Graham, 1965).

Nodule bacteria can multiply in media containing very little nitrogen, but they are usually depending on their nitrogenous reserves. Thus, while an initial culture of *rhizobium* has about 8 to 9 percent nitrogen on dry mass basis, a culture multiplying in a poor medium has only about 3.5 percent nitrogen (Burris and Wilson, 1945). In artificial nutrient media, nodule bacteria either do not fix molecular nitrogen at all or assimilate insignificant amount.

The concentration of yeast extract affects on growth and morphology of *rhizobia*. Skinner et al. (1977) have demonstrated distortion of cells at yeast extract concentrations of 0.35 percent and above and 1 percent favours growth and high
viability. Mannitol is the carbon source used for routine work with rhizobia, although not all strains can utilize it. Graham and Parker (1964) found that growth of fast growers with glucose, sucrose, arabinose, xylose and fructose; slow growers preferred arabinose, xylose and galactose. It may be advantageous to use arabinose as sole carbon source, even though it is 5-10 times more expensive than mannitol, and requires filter sterilization. It is probable that most sugars are not fully utilized, and slow growing rhizobia do not utilize sucrose (Burton, 1982). Kumar Rao et al. (1980) found that sucrose and in some cases commercial sugar served quite satisfactorily for shaker culture. Yeast extract mannitol medium is usually used, but calcium carbonate is omitted to avoid clouding the plates. Ertola et al. (1969) found that the addition of potassium nitrate increased growth and maintained the pH near neutrality. Balatti (1982) supported this result. Calcium carbonate is only required for long term storage on agar of fast growers and can generally be deleted for broth media (Vincent, 1970). Bacteria isolated from soil at Allahabad showed the highest in vitro nitrogen fixation efficiency in terms of nitrogen fixed per gram of carbon consumed in the presence of mannitol followed glucose and finally sucrose (Bahadur and Tripathi, 1980).

Nandi and Sinha (1974) experimented for mass scale production of Rhizobium inoculant in different concentrations
of yeast extracts in the fermentation medium. Mollases could serve as a superior carbon source for rhizobia growth. Liquid malt extract could be ranked in the list and the third one was the medium having sucrose and mannitol in combination.

Neutral baker's yeast solution 200 ml/l or oxford yeast extract 500 mg/l and Difco 250 mg/l served as a very suitable substrate for growth. The modified composition of medium comprised 10 g sucrose and 5 g mannitol and 250 ml yeast extract solution /l and served as a very suitable composition for rapid cell multiplication.

**Screening of Rhizobium strains.**

The first step in screening rhizobium strains following isolation and confirmation of purity generally is to test their parent host. This is done by growing plants in a nitrogen-free solution in jars under greenhouse conditions for a period of 4 to 6 weeks and measuring dry weight of total nitrogen content. The effective strain is determined by observing the size and colour of the nodules produced by each strain. The size and colour of the nodules will vary more or less with the soil even where inoculated seeds have been sown (Ham et al., 1971). The effectiveness of a nodule in nitrogen fixation and the extent of nodulation is determined by the compatibility of the host and the *rhizobium* strain.

Burton (1970, 1975a) used another method of screening good nitrogen fixing strains of *rhizobia* for their compe-
titiveness with highly infective poor nitrogen fixing strains in soils. Seeds inoculated with a peat base inoculum of the test strain are planted in sand impregnated with a massive inocula of the native ineffective rhizobia. Plant is sown aseptically in a nitrogen free nutrient solution so that growth depends on symbiotically fixed nitrogen. Dry weight and total nitrogen content of plants are measured after a growth period of 5 to 6 weeks. The dry weight of a plant is proportional to its nitrogen content (Erdman and Means, 1952). It seemed therefore, desirable to study the symbiotic effectiveness of the rhizobium strains, occurring in the area of investigation and study their cultural characteristics.

B. CULTURING OF ISOLATED Rhizobia IN CARRIER MATERIALS:

After nodule bacteria of leguminous plants had been isolated by Beijerinck (1888), the idea of using them for practical purposes became more popular. A greater demand is being made on alternative and inexpensive sources of nitrogen, many countries have turned to nodulated legumes to meet this need. Where rhizobium strains do not occur naturally in the soil they can be provided by inoculation of seed with selected strains.

There are three phases for the preparation of inoculants and a commercial preparation of nodule bacteria was first proposed by Nobbe and Hiltner in Germany (1896).
(a) Selection of strains of Rhizobium.
(b) The preparation of carrier cultures.
and, (c) Quality control of commercially produced cultures.

Studies of Legume Inoculants:

The first attempts to inoculate leguminous plants were made in the United States in 1896 by Duggar (1897). A fine peat crumble is used as a filler, being mixed with the cultures of nodule bacteria. Such preparations contain little moisture and are friable powders convenient to use. The seeds mixed with friable powders have to be inoculated by dusting. Nodule bacteria do not form spores and die quite rapidly in a medium without nutrients. Consequently their numbers gradually decrease in commercial preparations. Therefore, preparations are assumed to be active only for a limited time, usually the season in which they are used.

Most legume inoculated cultures are prepared by adding rhizobium broth cultures to finely ground carrier base materials such as peat, mixtures of peat with soil, compost mixtures, lignite (Sahni, 1977), coir dust (John, 1966), Charcoal powder (Newbould, 1951) or other organic materials. Peat or charcoal powder has proved to be the best. Agar broth and lyophilized cultures are recommended because of the very poor survival of these forms of the inoculum on seed (Date, 1968; 1970 and Vincent, 1970).
Peat cultures can be prepared in two ways —

(a) ground peat is mixed with a high count (> $10^9$ rhizobia ml$^{-1}$) broth culture in sufficient volume to provide the minimum numbers of rhizobium acceptable for use. Such cultures do not depend on further multiplication of the rhizobia in the peat and are prepared in bulk using non-sterile peat or as pure cultures by injecting packets of previously sterilized peat with the appropriate amount of culture. (b) Sterilized peat is inoculated with a small volume of culture and incubated to allow multiplication of the rhizobia in the carrier to ensure the survival of the rhizobia in peat in numbers high enough to meet a minimum standard of quality. For inoculation, individual strains were grown in a shake culture of yeast extract mannitol broth for 27 h, and the culture then added directly to surface sterilized seeds. The culture added to seeds as a thick slurry in 10 percent sucrose solution. In India, the use of lignite which Tilak and Subba Rao (1978) included in their comprehensive study of Indian carrier materials. The results of Iswaran and Apte (1970) show that charcoal, lignite or farm yard manure could very easily replace peat with addition of energy source and any one of the mixtures could be satisfactorily used as a carrier only to fast growing rhizobia depending on the availability of the material. Even after 8 weeks the minimum standard of 100 million rhizobia per gram of the inoculant is maintained by these mixtures which appear to be reasonable and practical to bring about
nodulation when the seed is inoculated. The wood charcoal powder as carrier material for the liquid broth of *rhizobium* on showing some promise (Newbould, 1951). The proportions of broth and carrier material are governed by the moisture holding capacity, but it is generally desirable to add broth to the point where the carrier remains friable without forming balls (Date, 1974).

**Studies on Seed Inoculation**

The objective in inoculating leguminous seeds or the soil where they are planted is to introduce sufficient viable nitrogen fixing *rhizobia* into the area where the seed will germinate to assure effective nodulation of the young seedling and an abundant supply of nitrogen for good crop yields. Seed inoculation is popular simply because it is convenient way of uniformly putting the nodule bacteria where specific *rhizobia* are absent or scarce. When a legume is inoculated successfully the resulting functional nodules quickly make the legume independent of soil nitrogen. Solid base inocula such as peat, lignite, compost, charcoal provide more protection to the bacteria than liquid lyophilized inocula (Vincent, 1958, 1965, 1974; Burton, 1964, 1976 a,c).

The leguminous seeds vary widely in size, shape, chemical composition, nature of the seed coat and other
factors. On an individual seed basis, big seeds usually receive a large inoculum than do small seeds. In the United States the normal rate of use of inoculum is 4.4 g/kg seed regardless of seed size. In Australia, larger doses of the inoculum are used on small seeds. Australians use less than 3 g of inoculum for 1 g of Soybean as compared to the 4.4 g/kg in the U.S.A. (Date and Roughley, 1977). The limited evidence available tends to suggest that big seeds need more rhizobia on an individual basis for effective nodulation than do small seeds.

There are three general methods of the application of rhizobial inoculants on seeds i.e. Dry or powder method, Sprinkle method and slurry method of which slurry method has shown encouraging results.

**Materials and Methods:**

A. **Isolation and culturing of Rhizobium strains in artificial media.**

The *rhizobium* strains of six leguminous crops had been isolated from the pinkish colour root nodules as per procedure laid down by Vincent (1970) and were used in the present experiment to observe their cultural behaviour in natural and artificial media. For this purpose, six isolates of each strains were maintained in the Fred's modified medium with frequent subculturing.
Growth in solid media: Growth pattern of rhizobium strains were examined on the different solid media in—yeast extract mannitol agar congo red medium (Hahn, 1966), yeast extract mannitol agar medium (Fred et al., 1932) and Glucose peptone agar medium (Vincent, 1970). The culture media after autoclaving, were poured aseptically (15 ml each) to sterilized petridishes. The pH of the medium was 7. The isolates were then inoculated by pour plate method near the burner. The petridishes were incubated at 28±2°C for 2-7 days depending upon the fast and slow growing rhizobium. The growth of colonies were studied in each media alongwith the gram—staining technique, colony character and morphological characteristics.

Growth in liquid media: The growth of the isolated rhizobium strains were tested in the liquid broth of Fred's modified medium of yeast extract mannitol. The liquid medium was sterilized in the autoclave and followed the inoculation of strains individually. The inoculated flasks were kept in the rotary shaker at 28±2°C for 3-6 days according to the fast and slow growers. The pH of the medium was maintained at 7. After attaining the proper growth, the medium had become cloudy and dense due to the multiplication of rhizobium.

The isolated rhizobium strains were identified and marked according to their places of collection and cropwise for convenient of reference in the subsequent experiments.
PLATE- I. Colony characters of 6 different strains of isolated Rhizobia.

PLATE- 2. Seed inoculation with Rhizobium of 6 different legume crops.
B. Screening of carrier materials for preparation of Rhizobium inoculants:

It is relatively easy to devise a substrate from a variety of carrier materials which support satisfactory growth and survival of rhizobia (Strijdom and Deschodt, 1976). The search for new carriers has revealed suitable materials which are usually cheap and readily available locally. For the purpose, wood charcoal powder, rice husk ash, farm yard manure, charcoal powder with farm yard manure (1:1) and (3:1) in proportion was taken for this experiment. The carrier materials were autoclaved individually and upon cooling, the liquid shake culture of rhizobium broth was added separately upto the point of water holding capacity (45 percent). The contents were mixed thoroughly and packed in polythene bags. Each of the sample was tested in plate culture method every 7 days till 14 weeks for total viable count. The results were recorded.

Preparation of Rhizobium inoculants:

In this experiment, the sterilized wood charcoal powder as carrier material was used and to it liquid broth of rhizobium was added. The proportions of broth and carrier material are governed by the moisture holding capacity, but it is generally desirable to add broth to the point where the carrier remains friable without forming balls (Date, 1974). The rhizobium inoculants for the respective legume
crops were made serial dilution $10^{-1}$ to $10^{-9}$ and plated on yeast extract mannitol agar medium in sterile petridishes. The petridishes were incubated at $28^\circ C$ for 6-10 days and studied the comparative survival rate of various strains for further experiment on the six legume crops.

**Efficiency test of Rhizobium inoculant:**

The prepared rhizobium inoculants were tested on six legume crops by Leonard jar method to check the efficiency of rhizobium (Leonard, 1943). The seeds were surface sterilized and inoculated by slurry method. The inoculated seeds were sown and the nitrogen-free nutrient solution was used in the glass jars. At the 45 days from the date of sowing, the plants were uprooted and compared the nodulation with the un-inoculated series. The results were recorded.

**Results:**

A. *Isolation and Screening of Rhizobium strains:*

Results of the experiments on the growth of rhizobium in the solid medium showed variation in rate of growth. This variation was observed even among the isolates of the individual crops. There was distinguishing colony character of the different rhizobium strains. The bacterial cells were almost similar morphologically in all the strains under investigation. Young bacterial cells were usually rod shaped.
measuring about 0.5-0.9 x 1.2-3.0 μm and were motile. However in old cultures nodule bacteria were found to lose their motility and became belted rods, showing alternate parts of differentially stained the cell protoplasm with aniline dyes. In gram stain, the rhizobium cells showed clear red in colour and were gram-negative rods. The pure rhizobium colonies were colourless on congo red yeast extract mannitol agar medium and on glucose-peptone agar medium, the growth of rhizobium was poor and no colour change of the media was observed. There was variation of colony character of rhizobium between the strains and the nature of growth. It was found that, growth was rapid in the strain no. A, B and D, while it was medium in C and slow in EF on solid and liquid media. The growth rates of both slow and fast developing strains showed enhancement by the addition into the media of compounds containing bound nitrogen in the form of yeast extract. Profuse growth of all the strains of rhizobium was obtained in yeast extract mannitol agar medium (Table 3 and plate 1).

The isolated rhizobium strains were screened out in order to obtain the efficient strains of the six legume crops and marked individual strains according to their places of collection as shown in Table 4.
Table 4 Showing identification of isolated *Rhizobium* strains as per their places of collection of different legume crops.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of the host’s</th>
<th>Strain number</th>
<th>Identification of individual <em>Rhizobium</em> strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tezpur</td>
</tr>
<tr>
<td>1</td>
<td><em>Pisum sativum</em></td>
<td>A</td>
<td>AT1a</td>
</tr>
<tr>
<td>2</td>
<td><em>Lens esculenta</em></td>
<td>B</td>
<td>BT2a</td>
</tr>
<tr>
<td>3</td>
<td><em>Cicer arietinum</em></td>
<td>C</td>
<td>CT3a</td>
</tr>
<tr>
<td>4</td>
<td><em>Phaseolus radiatus</em></td>
<td>D</td>
<td>DT4a</td>
</tr>
<tr>
<td>5</td>
<td><em>Glycine max.</em></td>
<td>E</td>
<td>ET5a</td>
</tr>
<tr>
<td>6</td>
<td><em>Vigna sinensis</em></td>
<td>F</td>
<td>FT6a</td>
</tr>
</tbody>
</table>
B. Screening of carrier material:
The wood charcoal powder was found best as a carrier material of *rhizobium* for maximum period of viability at 10^{-8} upto 14 weeks due to having 45 percent water holding capacity and is most suitable for the preparation of rhizobium inoculants as shown in Table 5. Moreover, the comparative survival rate of various strains of *rhizobium* in wood charcoal carrier material was tested on plating medium showed little variation on the number of *rhizobium* colonies formed in the serial dilution (10^{-6} to 10^{-9}), as shown in Table 6, Plate-2.

Inoculum strength of 10^{-6} was taken for different *rhizobium* inoculants for the six legume crops tested to study the efficiency on nodulation. The results showed that, the nodules were formed on the tap roots. The control series in the Leonard jar technique, the nodulation was not observed (as shown in Table-7). The effective inoculation was ensured only with a fairly large number of bacterial cells which varied considerably. The nodules were pink coloured due to the presence of leghaemoglobin. Nodules formed by active cultures of nodule bacteria usually have a whitish colour when young. At the post flowering period when the process of nitrogen fixation was terminated, the red pigment turned green. The change in colour from pink to green was observed at first at the basal region of the nodule and later extends to the apex. The transition from red to green pigment was accomplished at different
rates and with different intensities in different species of leguminous plants.

Table 6 Showing comparative survival rate of various strains of Rhizobium in wood charcoal powder carrier material on plating in YEMA*.

<table>
<thead>
<tr>
<th>Sl.</th>
<th>Name of the host's strain</th>
<th>Serial dilution of Rhizobium Inoculants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>1</td>
<td>Pisum sativum.</td>
<td>173</td>
</tr>
<tr>
<td>2</td>
<td>Lens esculenta.</td>
<td>168</td>
</tr>
<tr>
<td>3</td>
<td>Cicer arietinum.</td>
<td>154</td>
</tr>
<tr>
<td>4</td>
<td>Phaseolus radiatus.</td>
<td>178</td>
</tr>
<tr>
<td>5</td>
<td>Glycine max.</td>
<td>195</td>
</tr>
<tr>
<td>6</td>
<td>Vigna sinensis.</td>
<td>203</td>
</tr>
</tbody>
</table>

* YEMA = Yeast extract mannitol agar medium. Figures are average of 3 replications on number of colonies.
Table 7 Showing the efficiency of *Rhizobium* strains on the nodulation of respective hosts* in Leonard Jar assemblies.

<table>
<thead>
<tr>
<th>Sl.</th>
<th>Name of the host's</th>
<th>Average nodulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Un-inoculated</td>
</tr>
<tr>
<td>1</td>
<td><em>Pisum sativum.</em></td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td><em>Lens esculenta.</em></td>
<td>*</td>
</tr>
<tr>
<td>3</td>
<td><em>Cicer aritinum.</em></td>
<td>*</td>
</tr>
<tr>
<td>4</td>
<td><em>Phaseolus radiatus.</em></td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td><em>Glycine max.</em></td>
<td>*</td>
</tr>
<tr>
<td>6</td>
<td><em>Vigna sinensis.</em></td>
<td>*</td>
</tr>
</tbody>
</table>

Figures are average of 3 replications.

**Discussion:**

One of the major difficulty is the isolation and culturing of rhizobium strains in artificial media is the contamination by secondary organisms which hamper easy isolation of the strains in routine media. This primary obstacles led to the use of selective medium which enhances the growth of the rhizobium strains for slow and fast growers. A selective medium for the culture of rhizobium is very essential because it is easily overgrown by a number of microorganisms. In the present investigation, selective solid and liquid media were used. It is revealed from the results of the present investigation that, tested selective media induced good growth
of the rhizobium. In culture media, non motile and motile coccoid cells were observed. According to Nowak and Netzsch Lehner (1965) nodule bacteria usually exist as coccoid in the soil. Thickened, branched, pear-shaped or almost spherical formations also develop in bacteriological media and in nodules. In the root nodules, these forms were seen in the juice of the nodules.

Rhizobia grow readily in culture media containing carbon source. The bacteria formed colonies in the yeast extract mannitol agar medium and they were smaller rod shaped. It was also further observed that, these root nodule bacteria follows a definite cycle of growth form. Prazmowski (1890) who studied the development cycle of nodule bacteria both in nutrient media and in nodules and considered bacteroids to be inactive involutive forms. The results of the present investigation show that vigorous nitrogen assimilation in the plant coincides with the period of formation of the bacteroids. Therefore, it must be assumed that the bacteroids, especially those that were young, retain several active physiological functions. Fast growing rhizobia produced good growth on yeast extract mannitol agar plates as well as in liquid media within 3-5 days. Colonies were 2-4 mm in diameter, colourless, white, with moderate to copious gum strains producing little or no gum have smaller colonies. Slow growing rhizobia require 6-14 days to form 1-2 mm
colonies. The differential staining reaction observed in the old Rhizobia culture, and this may be due to deposition of fatty substances in the ageing bacterial cell which interfered staining action; while young rhizobium cells stained uniformly.

Siddaramaiah and Bagyaraj (1981) isolated rhizobia 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.

The use of rhizobium inoculant is an special advantage for the legume crop's production and in view of this, the isolated rhizobium strains were inoculated to the wood charcoal carrier material for making the seed inoculants. In the efficiency test of rhizobium strains to the specific host's, it was found that the pink nodules were formed in the root systems. Many investigators had confirmed that the nodules formed by effective strains of bacteria are pink due to the presence of leghaemoglobin (Virtanen, 1965). Leghaemoglobin apparently catalyses nitrogen assimilation (Virtanen and Laine, 1945). However, after the post flowering period the red pigment of the nodules turned into green colour. The change of colour from pink to green was observed at
first at the basal region of the nodule, which gradually extended to the apex. This transformation was different in different species of leguminous plants under investigation. This may be due to the oxidation of the red pigment to green colour. Appleby and Bergersen (1958) established that rhizobium bacteroids in the absence of oxygen may reduce leghaemoglobin. Significant differences were evident among legumes in the morphology of the nodules. The nodules were mostly club-shaped, branched, lobed, longer and baseball in structures.

Sawashe (1985) conducted a field experiment with different rhizobium strains of gram in order to study the nodulation pattern and the results thus obtained were statistically analysed and found to be significant. Results of the present experiment further reveal that all the strains were superiority over the control as shown in the Leonard jar method for testing the isolated rhizobium strains to the specific host's. Mehrotra and Lehri (1970) studied the isolation and selection of root nodule bacteria of major legume crops in Uttar Pradesh viz. berseem, pea in winter and dhaincha, sonai, soybean etc. in rainy season.

Lasting and Küüts (1966) found in field conditions that the success of inoculation depends on the number of nodule bacteria.
To distinguish active from inactive strains of nodule bacteria are manifest chiefly in a different distribution of the nodules within the root system. When plants are inoculated with active strains numerous nodules form on the tap root, with a few on the lateral roots. According to Radulovic (1966) this is connected with the growth rate of the root system, in particular of the root hairs on the tap root, through which the bacteria can penetrate. The time of growth of the root hairs decreases as the root develops, so reducing the possibility of nodule formation on the tap root. Inactive strains of nodule bacteria usually form small nodules scattered over the whole root system. The pattern of distribution and the shape, size of the nodules are primarily determined by the leguminous species. In the present investigation also variation in growth pattern of the *Rhizobium* in the culture media and on the host's was obtained which stands in conformity with the above workers.