CHAPTER

2 Legume (*Rhizobium*) Inoculants

2.1 INTRODUCTION

It has been estimated that above each hectare of land there are about 80,000 tonnes of nitrogen available in atmosphere. But atmospheric nitrogen is not available to plants or animals and they cannot make use of free nitrogen.

Rhizobia/Bradyrhizobia have the ability to fix atmospheric nitrogen in symbiotic association with legumes and certain non-legumes like *Parasponia* and *Tremma*. They normally enter the root hairs, multiply there and form nodules. The amount of nitrogen fixed varies with the strain of *Rhizobium*, the plant species and environmental conditions. Not all legumes fix nitrogen. The capacity to form nodules appears to be absent from the majority of the segregate family Ceasalpiniaceae. On the other hand nodulation, if not nitrogen fixation, with root nodule bacteria appear almost universal in the segregate family Mimosaceae and other Fabaceae, although only relatively small proportion of the total number of species in these two groups has been properly examined.

Legumes play unique role in plant kingdom and rank third amongst flowering plants in number of species they contain. Most of the species are trees, shrubs and woody vines. The cultivated species include mainly food, forage and pasture plants. Of the 1,300 species examined, representing all the three sub-families of leguminosae, about 14% were found to be non-nodulating (Allen and Allen, 1961).

Date (1970) reported that asymbiotic fixation of 10 kg nitrogen is equivalent to an application of 500 kg sulphate of ammonia. Since symbiotically fixed nitrogen is in organic form, there would be little loss and therefore, would be equivalent to an effective application of 100 kg nitrogen as ammonium sulphate. Different estimates of global N_2-fixation has been made from time to time. Burns and Hardy (1975) estimated that a total of 175 million metric tonnes of N is fixed per year through biological nitrogen fixation (BNF) of which legume N_2-fixation accounted for approximately 40%. It is believed that legume symbiosis contributes at least 70 million metric tonnes N per year; approximately one half of which is derived from the cool and warm temperature zones and the remainder from the tropics. BNF is an efficient source of N (Peoples *et al*., 1995). Values estimated for various legume crops are given in Table 2.1.

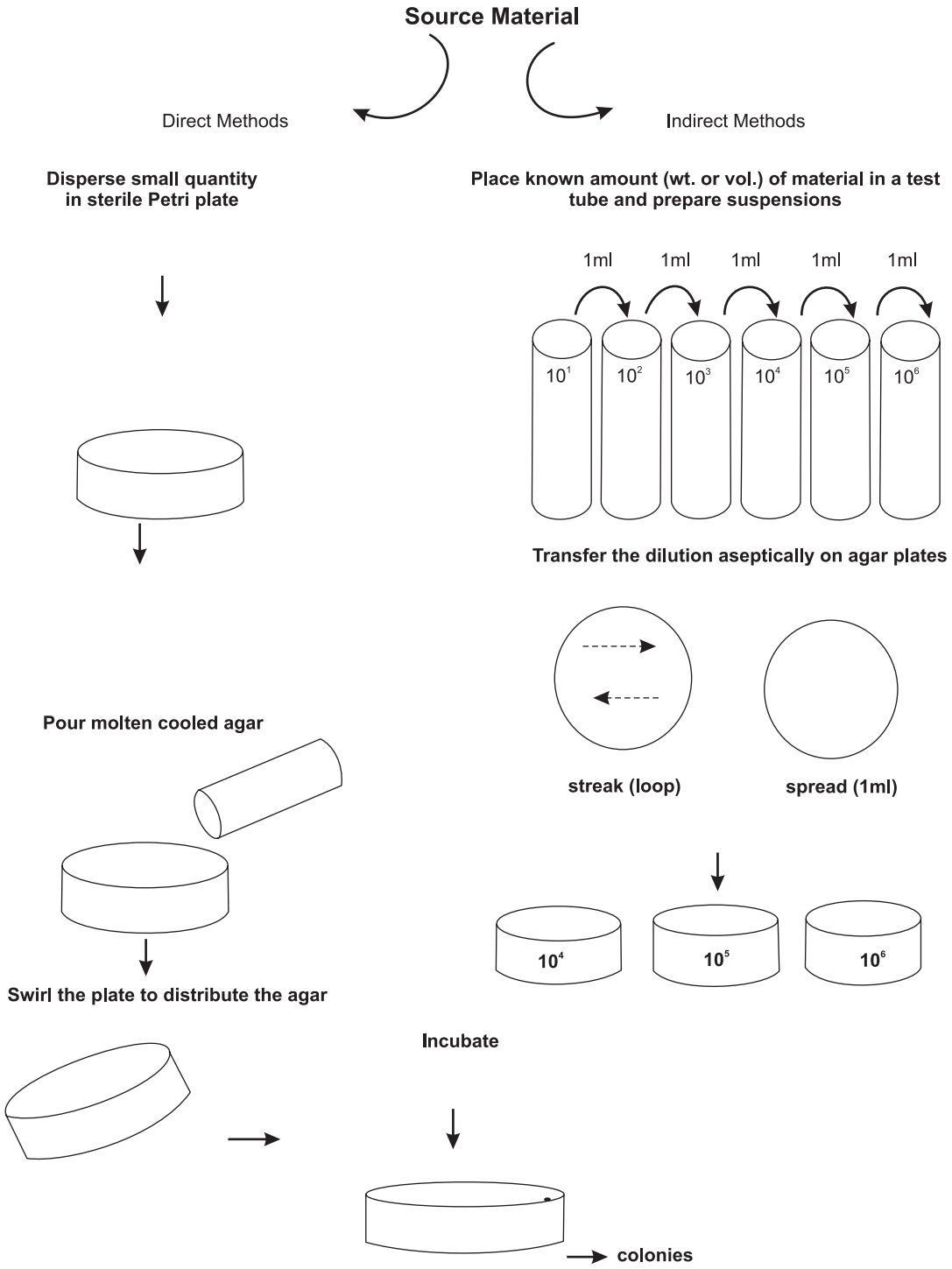

Fig. 2.1. Isolation of *Rhizobium*

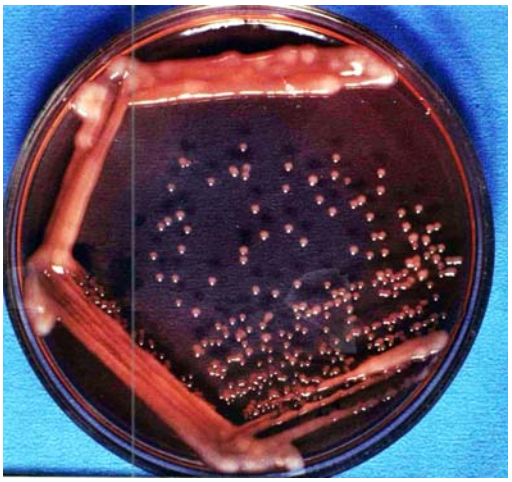

Fig. 2.2. Colonies of *Rhizobium*

ratios of the fast-growing species of *Rhizobium* are less closely related to the slow-growing species. The lack of homology between the DNA of *B. japonicum* and DNA of representative strain of *R. leguminosarum* is sufficient to justify the separation of rhizobia into two genera (Deley and Russel, 1965).

2.3 SYSTEMATICS OF *RHIZOBIUM*

The genus *Rhizobium* was established in 1889 by Frank (Fred *et al*., 1932) based on its ability to form nodules on the roots of legumes. The genus *Rhizobium* along with the genera *Agrobacterium* and *Chromobacterium* comprise the family Rhizobiaceae (Breed *et al*., 1957).

The leguminous species develop certain definite preference for certain kinds of nodule bacteria and vice versa. The earlier theories on the inter specificity of rhizobia to nodulate the species were soon tested and found incorrect. By 1900, it became apparent that there were groups of legumes, each of which were nodulated by a different type of *Rhizobium*. Each of these groups of legumes and their specific rhizobia constituted a cross-inoculation group and it was generally held (i) that with each cross-inoculation group a *Rhizobium* isolated for one legume member of the group would nodulate all other members of that group, and (ii) that rhizobia isolated from one plant in cross-inoculation group would not nodulate plants from other groups. For example, if a strain was isolated from lucerne (*Medicago sativa*), it could be expected to form nodules on *Melilotus* and *Trigonella* species, since these are also included in the same cross-inoculation group. But it would not form nodules on clovers or soybeans.

The taxonomy of the root-and stem-nodulating bacteria is in a state of transition. The classification of these organisms is based on plant infection into seven cross-inoculation groups to form nodules (Fred *et al*., 1932). They are listed in Table 2.2. This classification has been abandoned after extensive criticism (Graham, 1964).

Table 2.2. Cross-inoculation groups of *Rhizobium*

***Rhizobium* species**	**Cross-inoculation group**	**Legumes**
R. trifolii	Clover group	*Trifolium*
R. meliloti	Alfalfa group	*Trigonella, Melilotus, Medicago*
R. phaseoli	Bean group	*Phaseolus*
R. lupine	Lupine group	*Lupinus, Ornithopus*
R. leguminosarum	Pea group	*Pisum, Vicia, Lens*
R. japonicum	Soybean group	*Glycine*
Rhizobium sp. (miscellany)	Cowpea (miscellany) group	*Vigna, Arachis*

Another system of classification was proposed by Jordan (1984) in Bergey's Manual of Systematic Bacteriology (Table 2.3). He separated the root nodule bacteria into two genera, *Rhizobium* and *Bradyrhizobium*, based on the data on numerical taxonomy, mole per cent of G + C in the DNA, nucleic acid hybridization, cistron similarities, serological relationships, extracellular polysaccharide composition, carbohydrate utilization patterns, antibiotic sensitivities, SDS-polyacrylamide gel electrophoresis protein banding patterns, and rate of growth of rhizobia on laboratory media.

Table 2.3. Classification of root nodule bacteria

Genus	**Species**	**Biovars**	**Host legumes**
Rhizobium	*R. leguminosarum*	*viceae*	*Vicia*
	R. leguminosarum	*trifolii*	*Trifolium*
	R. leguminosarum	*phaseoli*	*Phaseolus*
	R. meliloti	—	*Medicago*
	R. loti	—	*Lotus*
	R. fredii	—	*Glycine*
Bradyrhizobium	*B. japonicum*	—	*Glycine*
	Bradyrhizobium sp.	—	*Cicer, Cajanus, Vigna*

Source: Jordan, 1984

The classification of root and stem nodulating rhizobia has been modified since 1984 and the changes have been made with more detailed studies on large number of *Rhizobium* strains from a wide variety of leguminous plants. At present only about 8-9% of the 14,000 or so known species of leguminous plants have been examined for nodulation, and less than 0.5% have been studied relative to their symbiotic relationship with nodule bacteria.

Since 1984, two additional genera, *Azorhizobium* and *Sinorhizobium* and five additional species *R. galegae, Sinorhizobium fredii* (formely *R. fredii*), *S. xinjiangensis, R. tropici* and *A. caulinodans* (stem- and root nodulating bacteria of *Sesbania rostrata* (Fig. 2.3) have been proposed and are found in most recent listing, i.e., in the Index of Bacterial and Yeast Nomenclatural Changes (Moore and Moore, 1992). The genera *Sinorhizobium* and the genus *Ensifer* belong to a single taxon (Young, 2003). *Azorhizobium caulinodans*, specific for stem nodulation in *Sesbania rostrata* can also nodulate *Phaseolus vulgaris*. *Aeschynomene aspera* and *A. indica* also form stem nodules in rice fields under waterlogged conditions. The stem nodulation is prevalent under submerged (flooded) conditions. *Neptunia natans,*

Fig. 2.3. Stem nodules in *Sesbania rostrata*

an aquatic legume is nodulated by *Allorhizobium undicola*. The Indian isolates of *A. undicola* from *N. natans* belonged to genus *Devosia* (Rivas *et al*., 2003). Root nodule isolates of *Acacia mangium* from Thailand and Philippines revealed that members of genus *Ochrobacterium* possessed the symbiotic ability to form nitrogen-fixing nodules. Since the preparation of that list, identification of significant genetic diversity in *B. japonicum* led Kuykendall *et al*., (1992) to propose a new species of *Bradyrhizobium* called *B. elkani*. Chickpea *Rhizobium* belong to a separate species, namely *Mesorhizobium ciceri*.

Sahgal and Johri (2006) outlined the current status of rhizobial taxonomy and enlisted 44 recognized species distributed among eleven genera, nine belonging to α-proteobacteria (*Allorhizobium, Azorhizobium, Bradyrhizobium, Devosia, Mesorhizobium, Methylobacterium, Ochrobacterium, Rhizobium* and *Sinorhizobium*), derived based on the polyphasic taxonomic approach. Species of *Parasponia* and *Trema* are the only non-legumes that form effective symbiosis with *Rhizobium* or *Bradyrhizobium*. Genus *Sinorhizobium* has been renamed as *Ensifer* according to the bacteriological code. *Burkholderia* spp. was described from nodules of South African legume, *Asphalathus carnosa* and *Ralstonia taiwanensis* in *Mimosa* nodules from Taiwan. Occurrence of *Ralstonia* from *Mimosa* nodules has been reported from India (Tripathi, 2002).

2.4 CHARACTERISTICS OF *RHIZOBIUM*

Many of the biochemical and physiological attributes like catalase, oxidase, urease, liquefaction of gelatin, indole production, reduction of methylene-blue, methyl-red, production of levan, Voges Praskauer reaction and fermentation of carbohydrates were

and are often oriented end-on to their host. Adhesion is initially mediated by the calcium (Ca)-binding protein rhicadhesin, or by plant lectins, with subsequent bonding via production of cellulose fibrils.

Rhizobia cause localized hydrolysis of the root-hair cell wall, and promote invagination of the host plasma membrane, with additional plant-cell material deposited about them as they infect. The enzymes involved in hydrolysis are cell bound and difficult to quantify, and several differ from those normally associated with plant infection. Rhizobial penetration causes root-hair growth at the point of infection to cease, and leads to root-hair curling, first visible some 6-18 hr after inoculation. The proportion of root hairs infected is low, and the percentage of these giving rise to nodules, highly variable. The root hair gets curled into a shepherd's crook due to penetration by several rhizobia, and the beginning of an infection thread takes place. Rhizobia, still encased within a plant-derived infection thread, move down the root hair to the root cortex. Cell division in the root cortex precedes their arrival and gives rise to the nodule primordia and in some legumes to an uninfected meristematic region. The spread of the infection thread among cells of the nodule primordium follows, with rhizobia released into their host by endocytosis. Rhizobia never gain free intracellular access. They are initially confined by the infection thread and later surrounded by the host-derived peribacteroid membrane.

Nodulation is usually evident 6-18 days after inoculation, but this varies with the strain and cultivar used, the inoculant density, and temperature. Initially, nodulation is heaviest in the crown of the root, with secondary flushes of nodules on lateral roots as the first-formed nodules senesce.

Infection and nodule formation are host specific phenomena. Legume species or cultivars which are nodulated by some rhizobial isolates are not nodulated by other isolates. It has been reported that host-bacterium symbiosis may be determined by the binding of host legume lectins (glycero-proteins) to characteristic carbohydrate receptors on the *Rhizobium* cell surface.

The lectin is present on root hair which serves as target cells for infection (Dazzo *et al.*, 1979). But not all legume seed lectins specifically recognize the corresponding rhizobial symbiont (Pueppke *et al.*, 1980), which emphasize that tests on lectin recognition hypothesis should not be restricted to only consider the classical haemagglutinating lectins from legume seeds, but rather should focus on whether root lectins bind specifically to rhizobial symbiont before infection.

A mature nodule consists of a central 'bacterial zone', which is surrounded by the nodule cortex. This zone is made up of host cells containing 'bacteroids' enclosed in membrane envelopes of host origin. Bacteroids are normally non-motile and are surrounded by three membranes. The volume and number of bacteroids have a direct relationship with the nitrogen fixation. Effective nodules invariably possess higher number of bacteroids than the ineffective ones (Chopra and Subba Rao, 1967).

Simultaneous with the formation of bacteroids, red pigment haemoglobin accumulates between the bacteroids and the membranous envelopes. This pigment is known as 'leghaemoglobin', the prefix 'leg' indicating its presence in leguminous root nodule. The globulin is produced by the plant, whereas the haem moiety is produced

by the bacterium (Legocki and Verma, 1980; Dilworth and Appleby, 1979; Leong *et al.,* 1982). Leghaemoglobin is only present in infected cells and its location is confined to the host cytoplasm (Verma and Bal, 1976). The amount of leghaemoglobin and bacteroid production has direct relationship with the amount of N_2 fixed by legumes (Chopra and Subba Rao, 1967).

2.5.1 Development of Nodule

During nodule development and maintenance, a number of plant and bacterial 'genes' are expressed sequentially in a well coordinated manner. Nodulins are nodule-specific proteins encoded by plant genes and produced during certain stages of nodule development (Legocki and Verma, 1980). Based on the possible functions, these may be divided into three categories:

1. Proteins responsive for maintenance of nodule structure
2. Enzymes induced in specific nitrogen assimilates and carbon metabolism of nodules
3. Proteins that support bacteriod function and thus facilitate nitrogen fixation (Fuller *et al.,* 1983). Based on the structural similarities of nodulins, two groups are recognized — one is C-nodulins or common nodulins as the name indicates — protein common to all nodules — and the other is S-nodulins or species-specific nodulins.

2.5.2 Host Specificity

Burton (1967) has attributed this to host specificity of rhizobia and occurrence of strains. Depending on strain-host specificity, the association may be effective, moderately effective and ineffective. The differences in nitrogen fixation due to the varietal differences have been reported by several workers. Due to strain specificity, only a few per cent of the infected root hairs may give rise to nodules.

The nitrogen fixed by *Rhizobium* depends upon the degree of closeness, compatibility between the host and bacterium and by the total environment, of which climate and host nutrition are also important. Now it has been established that different varieties of different legumes have specific bacterial preference. The association between genotype of nodulating bacteria and legumes in the formation of nodules and N_2 fixation has been referred to as being so intimate that one might conceive it as constituent of a new form of life possessing new and unusual properties (Fred *et al.,* 1932).

Host interaction ranges from complete lack of nodulation to varying amount and type of nodulation having varying degree of N_2 fixation. The reason may be attributed to genetic constitution of legumes as well as *Rhizobium* (Nutman, 1956). Nutman (1965) described two phases in symbiosis — one is nodule initiation, growth and its degeneration, and the other is interactions between bacteria and host in the infected cells of the nodule in which nitrogen is fixed. He also pointed out that the first phase is determined by sets of polygenes, some of which also control root growth, whereas second is characterized by major genes specifically affecting the intercellular compatibility between host and bacteria. Host genotype and bacterial strains compatibilities have been observed in different legumes.

2.6 *RHIZOBIUM* INOCULATION

Legume inoculation is a significant strategy for the manipulation of rhizobial microflora and improving crop productivity and soil fertility. However, in tropical soils where there is presence of adequate native rhizobia and high levels of mineral N, legume inoculation often fails. Thus, there is an urgency to identify conditions where inoculation is needed. Different diagnostic measures to decide about inoculation have been suggested by various workers. Inoculation should be carried out if;

(a) population density of species-specific rhizobia is low;
(b) the same or symbiotically related legume is not grown in the area in the immediate past history;
(c) wastelands have to be reclaimed;
(d) legume follows a non-leguminous crop in a rotation;
(e) soil is poor in mineral N (nitrate); and
(f) soils are acidic, alkaline and saline.

2.6.1 Selection of Rhizobial Strains for Inoculant Production

A large-scale screening should be carried out to identify ideal inoculant strain(s) for different legume crops. The criterion for selection may vary for particular soil types like acidic, sodic, sodic-saline, saline, nitrate-rich or heavy metal contaminated. Keyser *et al.,* (1992) considered following characters as desirable for a strain to be fit for use in commercial inoculants:

1. Ability to form nodules and fix N on the target legume
2. Ability to compete in nodule formation with populations of native rhizobia present in the soil
3. Ability to fix N across a range of environmental conditions
4. Ability to grow well in artificial media, in inoculant carrier and in the soil
5. Ability to persist in soil, particularly for annually regenerating legumes
6. Ability to migrate from the initial site of inoculation
7. Ability to colonize the soil in the absence of a legume host
8. Ability to tolerate environmental stresses
9. Ability to fix N with a wide range of host genotypes
10. Genetic stability
11. Compatibility with agrochemicals

2.6.2 Requirements of Rhizobia

Rhizobia are not very particular in their nutritional requirements. Synthetic media have never played a major role in culturing rhizobia, though it can be successfully grown in such media. For commercial production of cultures the utilization of cheap sources of special nutritive value is found to be more economical. Rhizobia, in general, utilize monosaccharides and disaccharides and to a lesser extent polysaccharides, alcohol and acids. Wilson (1952) and Graham and Parker (1964) observed that starch is not utilized by rhizobia. Mannitol is often employed in culturing rhizobia and the yeast extract is

The sterilized beads are transferred aseptically to the broth culture in the tubes and re-plugged and soaked for 1-2 hours. The tube is then inverted and the excess broth culture is allowed to soak into the cotton plug. The beads impregnated with rhizobia are transferred into the storage tube aseptically; the screw caps are replaced and tightened securely.

The tubes should be examined after a day or so to ensure that the silica gel is still blue. If it becomes pink or colourless, either too much moisture was absorbed when the beads were transferred or an improper seal might be permitting entry of moisture.

In regenerating a culture, the broth is inoculated with one or two beads. These are easily spread from the storage tube using a sterile needle with a slight hook. A week or more may be needed to obtain visual signs of growth. Once the broth becomes turbid, loopfuls should be streaked on presumptive test media to check for retention of purity.

2.7.5 Lyophilization

The cultures should be grown to pre-stationary phase on agar slants and suspended in preservation medium. About 0.1 ml suspensions should be dispensed in pre-sterilized cotton plugged ampoules. These are to be subjected to primary drying for 4 h. After making constriction in the centre of the ampoule, it is placed on the manifold and subjected to secondary drying for 4-8 hours. The ampoules are sealed under vacuum after drying for 2 h at 0.1 torr and stored in refrigerator at 4°C (Fig. 2.4).

Cultures can also be stored in liquid nitrogen (–196°C) for longer periods. Normally, the culture collection centres preserve the cultures by freeze drying and also by storing them in liquid N at –196°C.

Fig. 2.4. Lyophilized cultures

The merits and demerits of various methods of storage of bacterial cultures are given in Table 2.4.

Table 2.4. Merits and demerits of preservation methods of rhizobial cultures

Methods	Expertise and facilities required	Advantages	Disadvantages
Agar slopes in screw cap tubes or agar covered with paraffin oil	Basic microbiological knowledge and facilities for pure cultures (autoclaves, clean transfer area, tubes, media, etc)	Simplicity, low cost minimum facilities and expertise	Short storage time, increased chance of contamination and variants because of frequent subculturing
Porcelain beads	As above, plus availability of beads, suitable airtight containers and dry sterilization facilities for the silica gel desiccant	Low cost and longer storage time and therefore more time before re-beading, facilitates number of subcultures (i.e., one bead) from original	Not as long term as lyophilization and risk of contamination and variants during re-beading, time required for re-beading
Lyophilized or freeze dried	Basic microbiological facilities like lyophilizing equipment (vacuum pump, freezing facility under vacuum), ampoules, glass blowing burner, etc.	Once ampouled, minimum risk of variants of contamination, virtually permanent storage, can be stored at room temperature	Expensive for equipment and materials
Liquid nitrogen storage (–196°C)	Expertise as above, plus cryostat and liquid N source	Rapid operation, long term preservation	Very expensive, special precautions during freezing and thawing

2.8 REVIVAL OF PRESERVED CULTURES

Cultures preserved on agar under paraffin or glycerol could be revived by streaking a loopful of the culture over fresh medium. Culture from porcelain bead could be revived by transferring one bead into a tube containing broth, suitable for the growth of the concerned organism.

Freeze dried cultures are reconstituted by the following procedure:

The ampoule is surface sterilized with 70% alcohol and an incision by a glass knife is made at the middle, where there is cotton plug. At this junction a red hot glass rod is touched, to make a crack. This allows the air to enter after filtering through the cotton wool. At this place the ampoule is broken and the lower portion of the ampoule is treated as mini test tube. Immediately by using a Pasteur pippete, 0.2 ml broth is dispensed and by repeated sucking and releasing the liquid, the dried mass is dissolved. The suspension is plated over agar medium.

2.9 PRODUCTION OF *RHIZOBIUM* INOCULANTS

Having selected a suitable bacterial strain that can form effective nodules in association with selected legumes over suitable range of environmental conditions, it is necessary to lyophilize them to maintain their efficiency since subculturing at monthly intervals on artificial medium may lead to deterioration of the activity of the organisms. Whenever

Table 2.5. Optimum fermentation conditions for mass multiplication of *Rhizobium* strains

1.	Type of reactor	:	Stirred tank
2.	Type of operation	:	Batch
3.	Carbon source	:	Sucrose or Molasses (3-5 g/l)
4.	Nitrogen source	:	Corn steep liquor or Yeast extract
5.	pH	:	7.8 (controlled)
6.	Temperature	:	28°C
7.	Inoculum rate	:	10 % (V/V)
8.	Inoculum count	:	10^9 cells ml^{-1}
9.	Antifoam	:	LPG
10.	Aeration	:	0.5 VVM
11.	Agitation	:	Depends on the fermenter size
12.	Fermentation time	:	Variable (18-40 hrs)
13.	Cell processing	:	Centrifugation/membrane filtration followed by lyophilization
14.	ISI specification	:	10^8 g^{-1} carrier at the time of manufacture
15.	Shelf life	:	6 months from the date of manufature

When the number of rhizobia in the broth has attained the required standard (10^8-10^9 cells ml^{-1}), the broth should be added to the carrier for preparation of carrier-based inoculant.

2.9.1 Carriers for Rhizobial Inoculants

The medium in which rhizobia are allowed to multiply is an important factor in rhizobial culture preparation. The term 'carrier' is generally used for a medium which carries the live microorganisms. As per Bureau of Indian Standards (BIS) specification, the carrier should be in powder form and capable of passing through 150-212 micron (72-100 mesh) IS sieve. A good carrier material should:

1. have high water holding capacity;
2. be non-toxic to rhizobia;
3. be easy to sterilize by autoclaving or gamma irradiation;
4. be readily and inexpensively available;
5. provide good adhesion to seed;
6. have pH buffering capacity; and
7. have cations and/or anions exchange capacity.

Most of the peats meet these criteria and serve as base for inoculants. However, in India, high quality peat is not available although peat-like material of medium quality, designated as peat soil, is located in pockets in Nilgiri valleys in Tamil Nadu and in some parts of Rajasthan. The availability and characteristics of different materials in India, which are used as carriers, are given in Table 2.6 (Tilak, 1991). However, search for alternative carrier materials continues particularly in countries like India that have no natural deposits of good quality peat. Alternative inoculant carriers have also been listed in Table 2.6 (Brockweell *et al.,* 1995). In India, different carrier materials like peat, lignite, charcoal, rice husk, pressmud, vermiculite, soil and coir dust have been employed. Gels are among the most interesting alternative (Dawson *et al.,* 1989). In manufacturing inoculants, a period of "curing" (maturation) after addition of broth culture to carrier improves the quality of the product (Burton, 1976).

Table 2.6. Carriers used for *Rhizobium* inoculants in India

Carriers	Availability	Location	Water holding Capacity (%)	Organic matter (%)
Peat	5.5 million tonnes (total)	Ootacamund (Nilgiri Hills)	52-120	21-56
Lignite	3 million tonnes (annually)	Neyveli	92	28
FYM	Plenty	Everywhere	40-50	30
Charcoal	Plenty	Everywhere	90	77

2.9.2 Packing

After curing, the inoculant is packed in polyethylene bags (high density; 0.075-0.090 mm) or polypropylene bags. The packing material should have the following properties:

(i) should be stable towards gamma irradiation
(ii) should be autoclavable
(iii) should have high gas exchange capacity, and
(iv) should not allow high rates of moisture loss

2.9.3 Maturation and Storage

Inoculants must be incubated for a week in a room at an ambient temperature ranging from 25-30°C. During this period the bacterium multiplies and reaches to a required standard. The packets may then be stored in a cold room (4-15°C) till its use (Gibson *et al.,* 1975; Bottomley and Jenkins, 1983). It has been reported that nodule occupancy by inoculant strains declines in the years following establishment (Moenne-Loccoz *et al*., 1994). This differential behaviour could be because of inherent or environmentally induced genetic instability and it is difficult to distinguish between the two.

2.9.4 Factors Affecting Survival of Rhizobia in the Carrier

(a) Moisture content

Van Schrevan (1970) reported the effect of moisture content of the carrier-based culture upon the number of rhizobia. On the basis of several reports, it has been noted that the optimal moisture in sterilized carriers lies in the range of 40-60%.

(b) Aeration

Improved survival of rhizobia under the conditions of free access of air has been reported by several workers. A satisfactory container for rhizobial culture would be a packet of low density polyethylene film of 0.2-0.5 mm gauge.

(c) Temperature

The influence of storage temperature on the rhizobial population depends on the purity of culture and the moisture loss during the storage. Normally at room temperature (30°C), the rhizobial population decreases after 20-30 days of storage, whereas at refrigerated temperature (4-6°C) the decline in rhizobial population is rather slow. Van

Schrevan (1970) reported that the survival of *Rhizobium leguminosarum* in soil peat culture can be maintained even up to 4 years when stored at low temperatures (2-4°C). It is customary to pre-store the cultures at 25-30°C for 7-10 days before transferring to refrigerated temperatures.

Besides the above mentioned factors, the type of carrier material, type of neutralizing agents and sterilization also affect the growth and survival of rhizobia in the carriers.

(d) Liquid cultures

Currently, liquid inoculants are in practice by growing the rhizobia in yeast extract medium and additions like polyvinyl pyrrolidone (PVP), arabinose, alginate, glycerol, etc. are provided to keep the organism(s) viable for longer period (1 year or more) storage at ambient temperature.

2.10 QUALITY CONTROL OF RHIZOBIAL INOCULANTS

The quality of rhizobial inoculants is of great importance in ensuring field performance as well as for the commercial prospects of inoculant industry. Inoculant quality refers to the number of specific effective rhizobia in the inoculant. Quality control consists of strain testing and selection service, and control of the broth before incorporation into the carrier, of the carrier-based culture after maturation and control of cultures during the periods of storage.

Many brands and of varying quality of *Rhizobium* inoculants are marketed today in the country. Upto the final broth stage, each individual manufacturer does his own quality control. Evaluation of inoculant quality by enumeration of viable rhizobia is an accurate index of inoculating potential. Numerical considerations are of such significance in determining quality of inoculant products and their success in field that the necessity for quality control systems has been recognized in various countries. In India, Bureau of Indian Standards (BIS) (formerly ISI) listed the Indian standard specifications for *Rhizobium* inoculants in 1977 (IS: 8268-1976). This was revised from time to time by a committee. These specifications are given in Table 2.7. A regulatory act concerning bio-fertilizers has also been formulated.

Legume inoculant manufacturers normally test the broth culture for pH, agglutination, glucose-peptone agar, Gram-stained smear and total rhizobial counts. With a satisfactory preliminary result, broth is mixed with the carrier material. The final broth result based on satisfactory viable count permits manufacturer to blend the carrier material with the broth culture. This material is withheld from distribution until the viable plate counts on representative carrier samples of the batch have been obtained. The expiry time of 2 to 6 months is based on this test and confirmed by the plant nodulation results by using the following methods:

2.10.1 Large-Seeded Legumes

The following bottle jar (Leonard jar) method is used: A 20-oz bottle, which has its bottom cut off so as to provide a flat finely ground finish, is inverted on a quartz preserving jar in such a way that it sits firmly on the rim of the jar and has the lip of the

Seeds are sterilized, germinated and planted aseptically just below the surface of the moist sand. When the young seedlings are established, they are inoculated with 1 ml aliquots from the successive dilutions and the surface of the sand carefully covered with 1-2 cm layer of small, dried and sterilized gravel to prevent aerial contamination. From this stage the Petri dish or beaker can be left off to permit full plant development. The uninoculated and nitrate controls and units inoculated with counted suspension of the specific *Rhizobium* need to be provided.

Plants should be lifted and the roots washed free of sand at about 8 weeks or when the units known to have had rhizobia added are well nodulated. Again, if the effectiveness of fixation is also to be assessed, the nodule count will have to be delayed until there is sufficient differentiation between N-free and nitrate controls (seedlings solution containing 0.05% KNO_3 (see appendix 1).

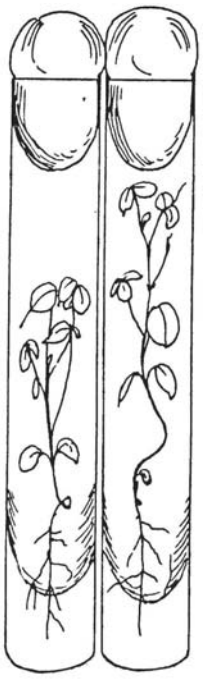

Fig. 2.7. Testing efficiency of rhizobial cultures using Gibson tubes

2.10.2 Small-Seeded Legumes

Test-tube studies : Surface sterilized seeds are grown on Jensen's nitrogen-free seeding agar slopes in 25 mm x 200 mm test tubes and inoculated with 1-2 ml of an active culture of *Rhizobium* strain concerned (Fig. 2.7). The tubes are incubated at 30°C under artificial light (2,000 lux). The root system is examined for nodulation from time to time.

2.11 METHOD OF APPLICATION OF *RHIZOBIUM* INOCULANTS

Proper use of inoculum, prompt sowing and soil improvement are essential to avoid undue loss of viability between inoculation and sowing, and permit rhizobia to multiply to a level that saturates root zone. The major goal of legume inoculation is to introduce efficient and competitive strains in large number which can survive and establish in the legume rhizosphere and colonize the roots promptly. Application of inoculant to the seed surface prior to sowing is the traditional, most commonly used and most user-friendly means of inoculation, although viability of rhizobia is subject to the hazards of drying, fertilizer contact, seed coat toxicity, incompatible pesticidal and mineral additives and adverse soil factors. There are numerous adhesives like gur or sugar, gum arabic, carboxy-methyl cellulose, cellofas A or vegetable oil which are suitable for attaching inoculant to the seed. Tenacity is the important characteristic of adhesives to ensure that inoculant is not lost from the seed during handling and passage through sowing machinery. The adhesive must be free from any preservative that might diminish the viability of rhizobia.

Usually, 400-500 g peat, lignite, or charcoal-based culture or 900 g^{-1} kg solid-based culture would be sufficient to the quantity of seed required per hectare.

The method of seed inoculation includes preparation of 10% sugar or pharmaceutical grade gum arabic or 1% carboxy methyl cellulose (CMC) solution. This solution is sprinkled on the seeds and the seeds are thoroughly mixed so as to have a uniform coating. A count of 1000 viable cells per seed is to be attained at the time of treating the seed and quantity of culture used is accordingly adjusted (Tilak, 1991). The seeds are spread uniformly for drying on a gunny bag or cement floor in shade avoiding direct sunlight. The seeds are sown immediately (Fig. 2.8).

Certain precautions are to be observed in the use of rhizobial cultures. The culture has to be used before the expiry date marked on the packet and the inoculated seeds are not allowed to come in direct contact with any pesticides or fertilizers. Although it has been shown that in most cases pesticides at recommended doses have no adverse effect on nodulation, it would be safer to use a double dose of the inoculant when fungicidal seed dressing is given. The mass multiplication scheme is represented in Fig. 2.9.

In order to protect rhizobia from effects of fertilizers, pesticides, acid and dry soils, pelleting of the seed is the most commonly used practice. The most commonly used pelleting agents are calcium carbonate (Iswaran *et al*., 1971a), rock phosphate (Iswaran and Jauhri, 1970), charcoal (Bhatnagar *et al*., 1980), talc, gypsum, betonite and neem leaves and neem cakes (Jauhri *et al*., 1981).

Roughley *et al*., (1960) has comprehensively described the inoculation and pelleting procedures. According to them, a good pellet should have following characteristics:

1. After mixing, the pellet should appear dry but without the loose lime left on the surface.
2. Pellets should be firm enough to drop on the floor without damage.
3. When dry, the pellets should withstand light rolling between the fingers.

Apart from seed inoculation, other methods of inoculation that have been tried are slurry or sprinkle method (Iswaran and Chonkar, 1971; Hely *et al*., 1980), soil inoculation (Hegde and Brahmaprakash, 1992), and inoculation of previous cereal crop (Gaur *et al*., 1980).

Problems like seed coat toxicity adversely affect the survival of *Rhizobium* on the seed. It was found that soaking the seeds in water for few hour removes the water-soluble toxic substances, and later coating the seed with the carrier-based culture usually results in establishing the requisite count of *Rhizobium* on the seed. Pelleting with finely processed lime (calcium carbonate) provides a fair protection against acidity in soil and fertilizers at the time of sowing. The pelleting material may also help the rhizobia to protect against the toxic effects of seed exudates.

Table 2.8. Influence of various pelleting agents on yield of pigeon pea

Sr. No.	Treatment	Grain yield (q/ha)
1	Uninoculated	12.4
2	Seed inoculated with rhizobial cultures	12.5
3	2 + Talc pelleting	14.7
4	2 + Charcoal pelleting	15.8
5	2 + $CaCO_3$ pelleting	16.0
6	2 + Rock phosphate pelleting	15.5

Source: Saxena *et al.* (1976)

2.12 FACTORS AFFECTING LEGUME – *RHIZOBIUM* SYMBIOSIS

2.12.1 Ecological Factors

Soil temperature at the sowing depth affects the efficiency of rhizobia. In tropical and subtropical countries like India, the soil temperature during summer month is as high as 45-50°C. High temperature affects root exudation, growth and survival of rhizobia, root-hair formation in plants and infection process. Since, the soil temperature under field condition cannot be controlled, temperature tolerant strains have to be used.

Moisture or water stress limits not only the survival of rhizobia but also their symbiotic association with the legumes (Venkateswarlu and Rao, 1987; Kulkarni *et al.,* 1988). Taneja *et al*., (1980) reported that water stress (-2 to -4 bars) resulted in decreased growth of *Rhizobium* strains. Salt tolerant *Rhizobium* strains are more tolerant to moisture stress (Mohammad *et al*., 1991) also.

The process of symbiotic nitrogen fixation is adversely affected by combined N, especially NO_3. The inhibitory effects of nitrate on infection, nodule development and N_2 fixation are well known (Saxena *et al*., 1996). Variations in the plant's ability to nodulate and fix N in presence of nitrate have been reported for strains of alfalfa (Heichel and Vance, 1979), pea (Nelson, 1987) and lentil (Saxena *et al*., 1996). The exact mechanism of inhibition is not very clear. The theories proposed ascribe the inhibition to a diminished supply of photosynthate available to nodules following its use for assimilation of NO_3

restricts the realization of the potential of N_2 fixation by legumes. The deficiency results in reduced yield, lower tissue per cent N and reductions in the proportion of N derived from fertilizers which occur with severe P deficiency (Thomas, 1995). Specific effect of P on the growth and survival of rhizobia and their capacity for nodulation and N_2 fixation has been reported (Singleton *et al.,* 1985). A striking example of the role of adequate P nutrition in enhancing N_2 fixation by soybeans is given by Cassman *et al*. (1993).

Among secondary nutrients, calcium and magnesium play an important role in legume-*Rhizobium* symbiosis. Calcium is involved in cell-wall synthesis of *Rhizobium*, and if grown in calcium deficient medium, it showed decreased attachment ability (Pareek, 1998).

Among micronutrients there is evidence that the requirement of boron for nodule development is similar to that for growth of the host (Munns, 1977). Likewise for nitrogenase activity, the need for Mo and Co far exceeds other plant requirements (Evans and Russell, 1971). Nickel is required for the synthesis of a functional hydrogenase (Patridge and Yates, 1982). Application of zinc in the form of zinc sulphate to soil upto a level of 5 ppm was stimulatory to lentil (Sukursha, 1976) and soybean (Tilak and Gangwar, 1973) in mollisols of Nainital Tarai, India. Zinc also improves nodulation and nitrogen fixation (Yadav *et al*., 1984). Zn^{++} is required for the production of indole acetic acid, which is important for nodulation in legumes.

Copper is required by many enzymes and at least thirty copper containing enzymes are known, all of which function as redox catalysts or dioxygen carriers (Weser *et al.,* 1979). The requirement for Zn, Ni, Cu and other heavy metal ions is generally at trace levels. However, above certain concentrations and over a narrow concentration range, their status can change from an essential growth promoting element to a toxin (Babich *et al*., 1982). Heavy metals at high concentrations inhibit growth of rhizobia in a nutrient medium and nodulation and nitrogen fixation by various legumes (Giller *et al*., 1993; Obbard *et al*., 1993). Costerton *et al*., (1981) postulated that the production of polysaccharides was a necessary adjunct to the survival of bacterial cells in polluted environments.

2.12.3 Biotic Factors

The presence of numerous parasites and predators poses problems in the establishment of rhizobial biofertilizer. The presence of antagonistic microflora has been postulated to affect the outcome of competition between the strains of rhizobia. Although some studies show that inhibition of *Rhizobium* by soil microorganisms may occur under laboratory conditions, the presence of inhibitory microorganisms in the legume rhizosphere has little effect on the nodulating ability of strains of rhizobia (Habte and Barrion, 1984). *Erwinia herbicola*, an epiphytic bacterium is commonly associated with seeds of *Medicago sativa.* It has been shown to inhibit nodulation by *R. meliloti* by producing toxin or by blocking rhizobial attachment sites on *M. sativa* root hair (Handelsman and Brill, 1995).

In the rhizosphere soil where rhizobia are present in large numbers, there is a build up of population of rhizobial phages (rhizophages). Negative interaction with them may

influence the establishment of *Rhizobium* strain (Barket, 1980). *Bdellovibrio*, an intracellular bacterial parasite of *Rhizobium* (Stolp, 1973), is capable of infecting and lysing large populations of rhizobia (Parker and Grove, 1970). The influence of *Bdellovibrio* has not been studied extensively, but seems to have little effect on competition and nodulation of rhizobia in soil (Alexander, 1977).

The size of populations of indigenous rhizobia has a major impact on the establishment and symbiotic performance of inoculant rhizobia, and it is possible to predict the performance of introduced strains using indices of the size of populations of indigenous rhizobia and soil N status (Turk *et al*., 1993). The competition among *Rhizobium* strains for nodulation of legumes has been extensively reviewed by Tilak and Saxena (1994).

2.13 RESPONSE OF LEGUMES TO *RHIZOBIUM* INOCULATION

Rhizobium inoculation improves the productivity of leguminous crop plants (Fig. 2.10). The efficacy of *Rhizobium* inoculation has been established in India beyond any doubt by the results of coordinated trials conducted by the Indian Council of Agricultural

Fig. 2.10. Field response of legumes to rhizobial inoculation
a. Groundnut (top: uninoculated control; bottom: inoculated with *Rhizobium*)
b. Soybean (top: inoculated treatment; bottom: uninoculated control)
c. Nodulation effect (left: uninoculated control; right: inoculated)

Research. The yield response varies with the inoculant strain, location and crop variety (Khurana, 1994). Soybean responds well to rhizobial inoculation at different locations (Annapurna and Balasundaram, 1995). The response of seed inoculation with *Rhizobium* culture on grain yield of gram, lentil, rajmash and soybean conducted at different locations is given in Table 2.9.

The response of seed inoculation with specific *Rhizobium* culture on grain yield of pigeonpea, greengram, blackgram, cowpea, chickpea and lentil at different locations under farmers field conditions is given in Table 2.9. The increase in grain yield due to rhizobial inoculation over uninoculated control ranged from 3.8 – 17.0 per cent depending on the soil type, crop variety and agro-climatic conditions.

Table 2.9. Response of rhizobial inoculation on grain yield of some pulses and soybean

Legume	Mean grain yield (kg / ha)						
	Coimbatore	**Delhi**	**Durgapura**	**Ludhiana**	**Pantnagar**	**Varanasi**	**Vamban**
Chickpea							
Inoculated	927	1329	1861				
Nitrogen (30 kg N/ha)	788	1347	1330				
Lentil							
Uninoculated control				748	1573		
Inoculated				854	2160		
Rajmash							
Uninoculated control						1716	613
Inoculated						1836	715
Soybean							
Uninoculated control		1307			1554		
Inoculated		1665			1552		

The figures are means of different varieties / rhizobial strains / years
(**Source**: Khurana, 1994; Annapurna and Balasundaram, 1995)

2.14 BENEFITS OF RHIZOBIAL INOCULATION TO SUBSEQUENT CROPS

The total nitrogen content of the poor soil increased as a result of rhizobial inoculation particularly when the crop was ploughed under the soil after the harvest. The amount of nitrogen fixed by various pulses and oilseed legumes is given in Table 2.10 (Peoples *et al.,* 1995). The extent of benefit can be judged by the yield of the subsequent crop. Experiments thus indicated (Table 2.10) that a well-inoculated legume crop might leave substantial amounts of nitrogen for subsequent crops (Subba Rao and Tilak, 1977; Wani *et al*., 1995).

The results presented in Table 2.10 show that in cereal-legume crop rotation system better *Rhizobium* symbiosis was obtained by double inoculation, i.e., when the preceding maize crop was also inoculated with the same *Rhizobium* strain, which was also used to inoculate the following legume crop of greengram (Gaur *et al.,* 1980). This was reflected in the greater volume of nodules, more dry matter of shoot and final grain yield. This could be attributed to better establishment of the introduced strain of *Rhizobium* in the soil by 2-fold inoculation, through the use of the rhizosphere of both the cereals as well as the legume crop involved in the crop rotation pattern.

Rhizobia possess nodulation (*nod* or *nol*) genes. These genes are inactive in the absence of legume. The root exudates components are identified as flavonoids which activate the regulatory protein NodD. This results in the transcription of the inducible *nod* genes. The products are essential for the biosynthesis/secretion of compounds like lipochitin oligosaccharides (LCOs) or Nod-factors. This compound acts as a major host-specific factor. Purified host-specific LCOs are able to induce several nodulation related effects. The genetic information for N_2 fixation in *Rhizobium* is envisaged through *nif* and *fix* genes. The legume contributes to the nodulation process by producing several nodule–specific proteins, designated as nodulins.

One of the factors essential for host range is the plant lectin. Diaz *et al*., (1989) demonstrated that transfer of the pea lectin gene to hairy roots of white clover resulted in nodulation and N_2 fixation of the transformed clover roots by *Rhizobium leguminosarum* bv. *viciae*, the bacterium specific for pea which is normally unable to nodulate white clover.

2.16 INTERACTION OF *RHIZOBIUM* WITH BENEFICIAL SOIL MICROORGANISMS

Studies on the associative effect of asmybiotic nitrogen fixers such as *Azotobacter* and *Beijerinckia* on symbiotic nitrogen fixation by legumes show that stimulatory and inhibitory effects are largely dependent on the strain of *Azotobacter/Beijerinckia* used. Sanoria and Rawat (1981) reported a significant increase in oven-dry weight of nodules and grain yield of chickpea when the seeds were inoculated with *Rhizobium* and *A. chroococcum* over the seed treatment with *Rhizobium* culture alone. The combined cultures had synergistic effect in increasing the phosphorus content of seeds also. Increased nodulation of chickpea was noticed (Table 2.11) when seeds of legumes were treated with *Rhizobium* sp. (cowpea miscellany) and *Azospirillum brasilense* (Tilak *et al*., 1981).

While studying the effect of plant growth promoting rhizobacteria (PGPR) on competitive ability of introduced *Bradyrhizobium* sp. (*Vigna*) for greengram nodulation, Gupta *et al*., (1998) reported that strains of *Enterobacter* increased the nodule occupancy of bradyrhizobial strains from 14 to 35 per cent.

There is much information from green house and field trials to show that VA-mycorrhizal fungi improve the growth, nodulation and nitrogen fixation in legume-*Rhizobium* symbiosis. Singh and Tilak (1989) demonstrated that the single VAM (*Glomus versiforme*) inoculation under field conditions produced significantly high grain yield and shoot dry weight of chickpea over uninoculated control in phosphorus-deficient sandy-loam soils (Table 2.12). Synergistic effects of VAM with rhizobia have been reported in several legumes (Fig. 2.12). To derive maximum benefit from dual inoculation with *Rhizobium* and VAM, addition of small amounts of P is also required (Manjunath and Bagyaraj, 1984; Subba Rao *et al*., 1986).

Saxena *et al.* (1997) reported that the nodulation competitiveness of *Bradyrhizobium* sp. (*Vigna*) strain S-24 was significantly higher (60-63%) in the presence of *Glomus mosseae*, *G. fasciculatum* and *Scutellospora calospora* when compared to treatment with single inoculation of S-24 (51%) in greengram.

Dual inoculation of *Rhizobium* with plant growth-promoting rhizobacteria viz. *Klebsiella planticola, Bacillus subtilis* and *Proteus vulgaris* has brought in positive response on grain yield of legumes like mungbean, chickpea, lentil, soybean, etc. (Anonymous, 2001). Strains of *Bacillus* sp. and *Enterobacter* sp. when inoculated with *Bradyrhizobium* sp. (*Vigna*) significantly increased the grain yield, nodule occupancy and N_2 fixation in mungbean (Gupta *et al.,* 2003).

2.17 EFFECT OF PESTICIDES ON LEGUME-*RHIZOBIUM* SYMBIOSIS

It was noticed that DDT when applied to soil did not affect the leghaemoglobin content of mungbeans up to 40 ppm. On the other hand, at 1-10 ppm levels the insecticides appeared to stimulate the pigment production. Similarly, application of lindane to soil at the rate of 1 ppm was not toxic to nodulation and growth of chickpea. Five ppm and above inhibited nodulation and grain yield (Magu *et al*., 1974). While studying the efficacy of various fungicides on pea crop, Singh *et al*. (1977) observed that the seed treatment with Dithane M-45 and Bavistin at the rate of 0.25 % (w/w) markedly increased the nodulation and nitrogen fixation by the legume. Some effects of insecticides in soil on nodulation of different legumes are presented in Table 2.13. Annapurna *et al.,* (2000) reported that thiomethoxan at recommended level can be applied in conjunction with *Bradyrhizobium* (IARI-1) inoculant for soybean.

Table 2.13. Effect of insecticides in soil on nodulation of legumes

Insecticide concentration	Nodulation effect
Lindane (2.2 g/kg seed)	Reduced nodulation
Isobenzene (2")	Decreased nodulation in lucerne
DDT (0.5 100 a.i.)	0.5 – 4.0 kg increased nodulation and nitrogen fixation in alluvial soil
Carbaryl (2.8; 14; 28)	No effect on soybean nodulation
Dieldrin	No effect on nodulation
Endrin	No effect on nodulation
Diazinon (80:120)	Decreased nodulation
Fonofos	Decreased nodulation
Disulfoton (1.7;8.4,16.8)	No effect on nodulation
Phorate (1.1 a.i.)	Increased nodulation
Chlorfenvinphos (1.1 a.i.)	No effect on nodulation
Trichlorfon	No effect on nodulation

Source: Gaur (1980)

In the light of the remarkable gains in crop production, which are possible through the use of effective cultures of the rhizobia, one wonders why all legume seeds are not inoculated. Yet, it is known that in some areas the soils do harbour an abundant supply of effective root nodule bacteria for the crops which are grown.

The legume productivity can be increased by exploiting synergistic effects of dual inoculation of rhizobia with other beneficial soil (rhizosphere) microorganisms. In view of the greater emphasis laid on the concept of sustainable productivity the present applied research in BNF should not only be directed towards *Rhizobium* inoculation technology but other options, such as manipulation of agronomic practices to favour BNF or genetically altering the plant to increase the symbiotic activity should also be looked into.