Symbiosis, 2 (1986)
Balaban Publishers, Philadelphia/Rehovot

Selection and Uses of Symbiotic Legume Bacteria. State of the Art and Potential Applications*

JEAN-CLAUDE CLEYET-MAREL and XAVIER PINOCHET

I.N.R.A. Laboratoire de Recherches sur les Symbiotes des Racines, 9, place Viala, 34060 Montpellier Cedex, France
Tel. 67-61-23-04 Telex INRAMON 490818F

Abstract

Legume inoculation is a way of assuring that strains of *Rhizobium*, appropriate for the cultivar being seeded, are present in the soil at the proper time and in numbers sufficient to assure quick and effective infection and efficient subsequent N_2 fixation. The most common commercial inocula in use today is the mixture of peat and a selected strain of bacteria. In the last years, new developments able to increase inoculum quality and facilitate their better use have been developed.

Keywords: *Rhizobium*, legume, inoculation, symbiosis

1. Introduction

Nitrogen is one of the elements essential to the synthesis of amino acids which, in turn, are used by the plant to form protein. Plants primarily take nitrogen in the ionic form of either ammonium (NH_4^+) or nitrate (NO_3^-).

Leguminous plants are able to utilize nitrogen derived from the symbiotic (mutually — beneficial) relationship they form with *Rhizobium* bacteria. This phenomenon, symbiosis, is extremely important and the value of this "free" fertilizer N_2 can be placed in global perspective if one considers that an estimated 50 million tons of N are manufactured industrially each year,

*Reviewed

0334-5114/86/$03.00

low infectivity or effectiveness, competition among strains of rhizobia, or poor survival in the soil.

2. Evaluation of the Need for Inoculation

The requirements for a successful symbiosis are definable and outwardly simple. They are: a reasonably healthy host-legume, capable of synthesizing enough carbohydrate to satisfy its own and the bacterial energy requirement, a strain of *Rhizobium* compatible with the host, effective in assimilating atmospheric nitrogen and, an environment conductive to the vigorous growth of both organisms.

Bacteria of the genus *Rhizobium* are fairly widespread in nature. However, there are several species within the genus and many strains have been identified within some of the species. Data from temperate zone studies and, more recently, some that involved tropical species, indicate that some strains of *Rhizobium* tend to be rather host-specific. That is to say, a given species or, even, cultivar of crop legume may be effectively infected and may carry on an efficient symbiosis only with a particular strain of one species of *Rhizobium*. In agricultural practice, several situations may be encountered.

1. The associated bacteria is present.

If it has a good ability to fix nitrogen, there is no problem,the plant is well fed. In France peas, clover, faba bean, alfalfa on alkaline soil are examples. If rhizobial strains are deficient in any way, we can introduce through inoculation a better strain of the same specificity, but we have to resolve the competitive problem for nodule formation. This situation has been found in France for chickpea, for the soybean in USA and for beans in several tropical countries.

2. The associated bacteria is absent.

Absence of associated bacteria could result from the environmental conditions that could not ensure the bacteria's survival, e.g. lupin in alkaline soils, alfalfa in acid soils (Amarger, 1980). This situation occurs also when the plant and its associated *Rhizobium* have never been cultivated in the considered area. This is the case for the soybean in France (Lagacherie and Obaton, 1973). In those situations, inoculation is needed.

3. Selection of Effective Strains

Criteria for selecting superior strains must be established and these should be considered when evaluating a particular strain. Current thought establishes three criteria as basic to a strain selection program.

1. Effectiveness in nitrogen fixation.

Effectiveness is the first criteria of any one who is attempting to increase legume yield through symbiotic nitrogen fixation (Vincent, 1970) and many quantitative tests have been developed to measure the reduction of dinitrogen to ammonia. Effectiveness is probably the easiest parameter of nitrogen fixation to evaluate and one should use simple tests such as dry weight yield or total-N-content (Wacek and Brill, 1976). Some other tests involve expensive equipment and highly trained personnel to operate the equipment and interpret the data (Hardy et al., 1968; Montange et al., 1981).

2. Competitive ability in the presence of a native rhizobial population.

An important characteristic in strain selection is the ability of a strain to successfully compete with the less effective native rhizobia for nodule sites. Ideally, one should be able to introduce a highly effective *Rhizobium* strain to a field where a less effective native population is present (Means et al., 1961; Ham et al., 1971; Amarger, 1981). The *Rhizobium*-legume interaction, itself, may dictate whether a superior strain will compete with the native rhizobial population (Materon and Vincent, 1980).

3. Ability to survive in various environmental conditions.

Persistence in the soil is a characteristic with which strain selection should be concerned. The effect of temperature on growth and persistence of *Rhizobium* has been well documented (Brockwell et al., 1972; Munevar and Wollum, 1981). In high temperature elevations the rhizobia fail to persist.

It has been shown that particular strains of *Rhizobium* were associated with particular soil types (Damirgi et al, 1967). This phenomenon may be related to the pH of the soil, the organic content, or the ionic composition.

High levels of nitrogen, moisture levels or pH extremes are additional criteria which should be considered in selection for strain survival.

It is recommended that strains be isolated and selected from the environment where they are intended to be used (Date and Halliday, 1979).

4. How to Produce Inoculum

The selected strains are multiplied in a fermenter on artificial media that ensure the best growth of bacteria. Systematic controls are used to eliminate contaminated cultures with foreign micro-organisms. Sterile peat bags are used to package the inoculum. This bag content is mixed with seeds just before sowing. In France, very strict quality norms have been edicted.

Recommendations as to standards are 5000 rhizobia per seed for lucerne and 10^6 per seed for soybean. Lucerne inoculation is made at the time of

sowing by mixing seeds (20 kg.ha^{-1}) with 200 g of peat inoculant containing at least 5×10^{10} rhizobia. For soybean, 400 g of peat inoculant containing 4×10^{11} rhizobia are mixed with 80 kg of seeds.

When one of the situations previously described is identified, inoculation can significantly increase the yield. Many difficulties may occur from the moment of fermenting to the moment of applying the inoculum in the field. During growth in broth medium, contamination must be avoided. Even a low number of fast-growing contaminants early in the process can suppress the growth of rhizobia. This event can occur with fast-growing rhizobia and the slow-growers are more susceptible. Several legumes can be nodulated both by fast and slow growing strains (e.g. *Glycine max, Cicer arietinum*). When given the choice after strain selection, between a fast and slow growing strain, the fast strain is the means of reducing the time needed to obtain sufficient growth and is less risky for an occasional contaminant.

Rhizobium populations have to survive at a high level inside their package substrates. Peat properties which ensure rhizobia survival are very different. Preliminary studies are necessary during several months or years to prove the possibility of their storage. In France, commercial incula can be stored for one year without damage.

5. Rhizobium Inoculation

Agronomic utilization of inoculants requires a production technology where rhizobial strains can be processed into inoculants with a high viable rhizobial density. Current methods involve some form of cultured suspension of bacteria applied to the seed just before planting. The number of viable rhizobia that needs to be applied on the seed varies with the size of the seed. It is recommended to inoculate the seed just before planting. No more than 4–6 hr should elapse before inoculated seed are used. The seed should be visibly moist but should not clump (for small seed) or drip, if large. Water helps the inoculum carrier to stick better to the seeds and each seed should have some black specks on it, indicating presence of the carrier. Direct sunlight on inoculated seeds must be avoided because of the sterilizing effect of the ultraviolet rays which are lethal to the rhizobia. Treatment of seed with biologically active materials (fungicides, insecticides, herbicides) will destroy *Rhizobium* and have to be avoided when their use is not essential.

Various authors have suggested that the inoculant for chemically-protected seeds should be added directly to the soil before planting or at the time of planting. However, broadcasting the mixture over the soil surface and then

covering it lightly by cultivation, necessitates two special field operations. It is not feasible in a hot dry climate, since it is likely to result in rapid drying of the scattered mixture and a high dispersion and mortality of bacteria before they are incorporated into the moist soil.

6. Pelleted Seed

The idea of pelleting seed has been tested many times and in many ways. Basically, the concept is to protect the inoculum from the toxic effects due to acid soil and to add the appropriate rhizobia to the seed at the factory or main distribution center (Brockwell, 1962). The idea of enclosing the seed in a coating of lime or in a coating of phosphate along with an inoculum, facilitate protection of the bacteria and seed, plus amelioration of adverse conditions in the soil adjacent to the seed. Pelleting is a good alternative to broadcast lime or rock phosphate only when inoculation and pelleting techniques are satisfactory (Lowher, 1975).

7. Response to Inoculation

Population densities in soil and rhizospheres

If the inoculation has been done properly and the seed sown promptly, the bacteria will begin to multiply shortly after they are placed in the soil. Fluorescent antibody (FA) techniques, which allow the identification and enumeration of specific strains of *Rhizobium* directly in the environment (Crozat et al., 1982), provide a unique tool for the study of population changes of *Rhizobium* in soil and in the rhizosphere. By studying survival kinetics of *Bradyrhizobium japonicum* strains introduced into soils using F.A. techniques, we observed during the incubation that all the different populations of a strain reached the same survival balance level, generally about $10^3 - 10^4$ *B. japonicum* g^{-1} soil (Crozat, 1983). The equilibrium threshold may correspond to the number of ecological sites available to rhizobia. Environmental factors which affect the nodulation process are antagonistic microorganisms and heavy metals such as copper which is found after vines are pulled out. High levels of nitrates, temperature and water potential influence both nodule establishment and nitrogenase activity (Trinick, 1982).

Nodulation capabilities in the field

Determining the nodulating capabilities of a strain, involve the selection of a site or sites for field work. To evaluate nitrogen fixation, it is necessary to select a site where no nodulating strain is present for that particular legume.

To test the competitiveness and the persistence of a particular strain, a native population of rhizobia is needed.

Nodulation is assessed at ten to twenty weeks, depending on the legume and the seasonal conditions, by sampling a number of plants in each treatment. Nodule number is a valuable criterion in the first six to eight weeks of plant development but is of very little use thereafter. At that point the best criterion is nodule dry weight and dry weight of the whole plant.

8. Potential Applications

In the last few years, new developments able to increase inoculum quality and to get a better utilization of them have occurred.

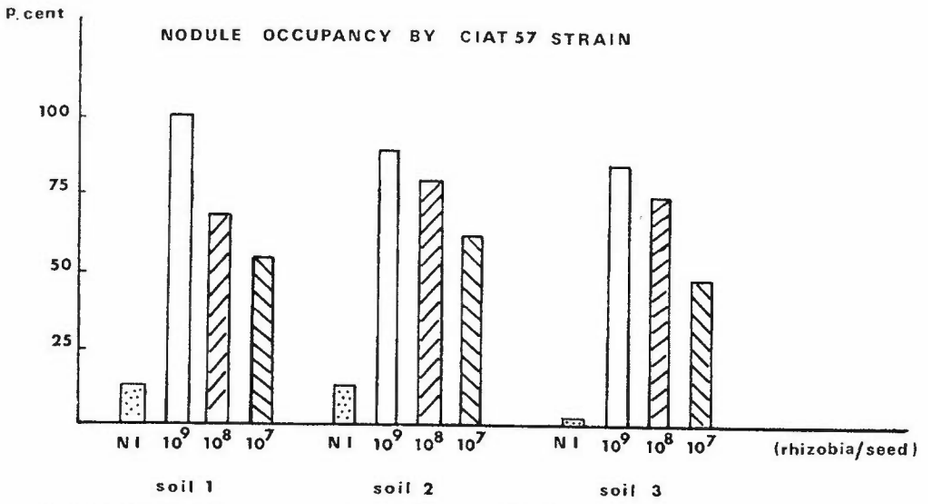

Figure 1. Inoculation response in 3 different soils for *Phaseolus vulgaris* inoculated with *Rhizobium phaseoli* strain CIAT 57 at 3 concentrations. NI: Non inoculated plot.

From a methodological point of view, the use of immunochemical technique is a mean to assist inoculum production in fermentors and to follow populations introduced in the field (Schmidt, 1974; Cleyet-Marel et Crozat, 1982). Today we mainly use the immunofluorescence technique. With this technique we can detect primary contamination in the fermenter and can quantify with good precision level, rhizobia in laboratory conditions, in the soil and on the roots. We are also able to recognize a strain in free living conditions or in a nodule. With this speedy and easy technique, we are also able to get results in a few hours. Competitivity measurements (Fig. 1) for

nodule formation in the presence of another strain of the same host specificity is also possible (Cigales-Rivero, 1984; Arsac, 1983). Immunoenzymatic tests (Elisa), can also be used and are well adapted in identifying the implantation rate of an inoculated strain in soybean nodules (Fernandez-Flouret, 1984). Similar results have been found in bean nodules and with immunsera obtained with soluble antigens from *Rhizobium phaseoli* (Fleurence, 1985), (Fig. 2). Elisa technique can be also used with chickpea and clover nodules (Arsac, 1983; Martensson and Gustafsson, 1985).

With the Elisa test, an expensive microscopic material is not necessary. Very often the coloration intensity of the enzymatic reaction is sufficient to give a visual result.

From the methodologic point of view, mathematical and computer tools can be used to study more precisely the growth rate and variations during the culture for better yield. We are also able to make progress through biological material. We must exploit the natural genetic variability inside rhizobia and find new strains. Besides nitrogen fixing levels and competitiveness for nodule formation, other selection criteria can be taken into account. It could be:

- the strain's growth rate in a defined medium which could increase the productivity in inoculum business and decrease the risk of contamination. This latter point is very important in tropical countries with low technical possibilities. Since 1982, fast growing strains nodulating with soybean have been isolated (Keyser et al., 1982).
- Adaptation criteria to environmental condition can be found such as the ability to nodulate with nitrates at low temperatures (McNeil, 1982). This has a practical interest because we meet these situations in France and industrial countries. These selected strains can be also useful for scientists to study mechanisms through which the environmental factors influence nodule formation.

9. Conclusion

Some basic principles concerning inoculation of legumes are well documented (Vincent, 1970).

Legume inoculation is a good example of a very successful bacterial introduction in soil with economical benefits for farmers. Greater efficiency may result if:

- inoculum standards are increased
- inocula have greater longevity
- more reliable inoculation techniques are introduced.

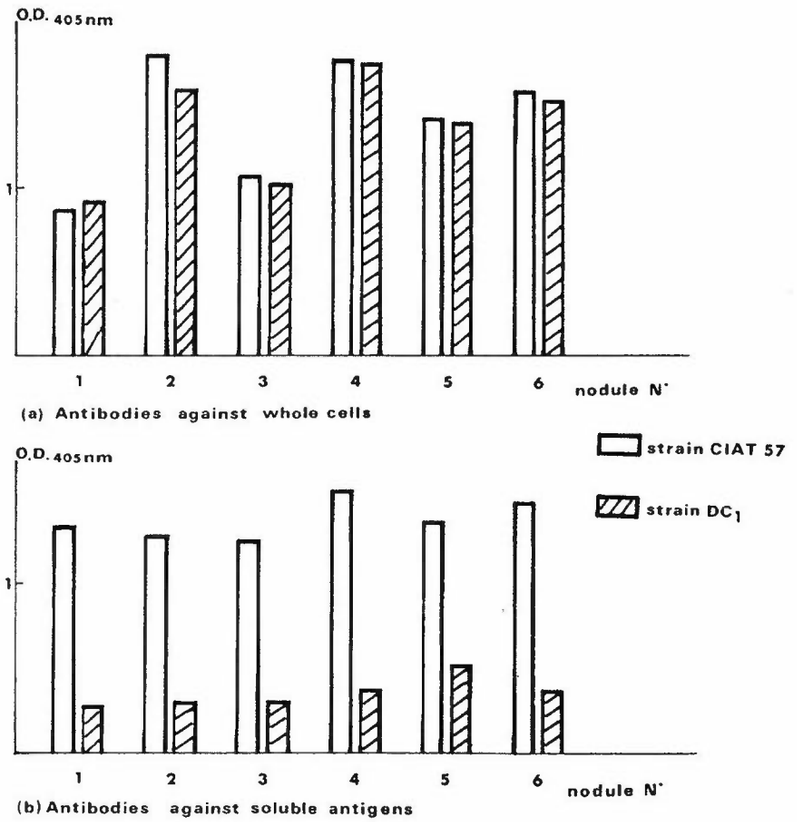

Figure 2. Elisa test used to characterize two strains of *R. phaseoli* in nodules if *Phaseolus vulgaris*. Absorbance values (A405) for homologous and non homologous reactions between nodule antigens and antiserum prepared against whole cells of *Rhizobium* (a) or with antiserum prepared against strain specific soluble antigens (b).

Availability of materials (e.g. fermenters, type of carrier) and an experienced and well qualified microbiologist are factors governing the production of legume inoculants. Moreover, the leader of an inoculant production unit has to carry out supporting research about strain selection and maintainance, quality control of each batch of inoculant and *Rhizobium* ecology in the field.

However, it must be kept in mind that inoculation of legumes is not a panacea. It represents only one aspect of a total management program.

Where inoculum is inexpensive relative to the expected gain, inoculation is often practiced as a form of "insurance". Under tropical or sub-tropical conditions, the result may not be one of success or failure, but rather of modest gains in infectivity of an introduced strain or effectiveness in fixing a greater quantity of nitrogen. The final decision on the value of inoculation at any given site must be an economic one.

With inoculation, the microbial partner of the symbiosis is involved but there is also a potential progress through plant selection. Several groups are working to select legumes for nitrogen fixing ability (Imsande, 1985; Phillips and Teuber, 1985).

REFERENCES

Amarger, N. 1980. Aspect microbiologique de la culture des légumineuses. *Sélectionneur Francais* **28:** 61–66.

Amarger, N. 1981. Selection of *Rhizobium* strains on their competitive ability for nodulation. *Soil Biol. Biochem.* **13:** 481–486.

Arsac, J.F. 1983. Etude de la symbiose *Rhizobium-Cicer arietinum* (pois chiche): aspects microbiologiques. DEA d'agronomie, USTL — INRA Montpellier.

Brockwell, J. 1962. Studies on seed pelleting as an aid to legume seed inoculaton 1. coating materials adhesives, and methods of inoculation. *Aust. J. Agr. Res.* **13:** 638–649.

Brockwell, J., Bryant, W.G., and Gault, R.R. 1972. Persistence of *Rhizobium trifolii* in association with white clover at high elevations. *Aust. J. Exp. Agric. An. Hasb.* **12:** 407–413.

Cigales-Rivero, M. 1984. Apport des techniques immunochimiques à la connaissance de l'écologie de *Rhizobium phaseoli.* Thèse Docteur Ingénieur, INRA-ENSA, Montpellier.

Cleyet-Marel, J.C. et Crozat,Y. 1982. Etude écologique en immunofluorescence de *Rhizobium japonicum* dans le sol et la rhizosphère. *Agronomie* **2:** 243–248.

Crozat, Y. 1983. Caractérisation du pouvoir saprophyte des souches de *Rhizobium japonicum* dans le sol et la rhizosphère. Thèse de Docteur Ingénieur, Université Lyon I, INRA. Montpellier.

Crozat, Y., Cleyet-Marel, J.C., Giraud, J.J., and Obaton, M. 1982. Survival rates of *Rhizobium japonicum* population introduced into different soils. *Soil Biol. Biochem.* **14:** 401–405.

Damirgi, S.M., Frederick, L.R., and Anderson, I.C. 1967. Serogroups of *Rhizobium japonicum* in soybean nodules as affected by soil types. *Agron. J.* **59:** 10–12.

Date, R.A. and Halliday, J. 1979. Selecting *Rhizobium* for acid, infertile soils of the tropics. *Nature* **277:** 62–64.

Fernandez-Flouret, D. 1984. Apport de l'immunofluorescence et des tests immunoenzymatiques (ELISA) à l'ètude du mécanisme de compétition envir souches de Rhizobium — D.E.A. Physiologie de la nutrition des végétaux — USTL-INRA-Montpellier.

Fleurence, J. 1985. Apport de l'électrophorèse et des techniques immunochimiques á l'identification des souches de *Rhizobium phaseoli* DEA d'agronomie. USTL-INRA Montpellier.

Ham, G.E., Cardwell, W.G., and Johnson, H.W. 1971. Evaluation of *Rhizobium japonicum* inoculant in soil containing naturalized population of rhizobia. *Agron J.* **63:** 301–303.

Hardy, R.W.R., Holsten, R.D., Jeckson, E.K., and Burns, R.C. 1968. The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. *Plant Physiol.* **43:** 1185–1207.

Imsande, J. 1985. Plant genotype and the control of nitrogen fixation in soybean. *Nitrogen Fixation Research Progress Abstracts* 1985:34.

Keyser, H., Bohlool, B., Hu, T., and Weber, D. 1982. Fast-growing *Rhizobium* isolated from root nodules of soybean. *Science* **215:** 1631–1632.

Lagacherie, B. and Obaton, M. 1973. L'inoculation du Soja: résultats d'essais et organisation future du travail. *C.R. Acad. Agric. Fr.* **59:** 67–77.

Lowher, W.L. 1975. Pelletting materials for oversown clover. *N.Z. J. Exper. Agric.* **3:** 121–125.

McNeil, D.C. 1982. Variations in ability of *Rhizobium japonicum* strains to nodulate soybean and maintain fixation in the presence of nitrate. *Appl. Environ. Microbiol.* **44:** 647–652.

Martensson, A.M. and Gustafsson, J.G. 1985. Competition between *Rhizobium trifolii* strains for nodulation, during growth in a fermenter, and in soil-based inoculants studied by Elisa. *J. Gen. Microbiol.* **131:** 3077-3082.

Materon, L.A. and Vincent, J.M. 1980. Host specificity and interstrains competition with soybean rhizobia. *Field Crops Res.* **3:** 215–224.

Means, V.M., Johnson, H.W., and Erdman, L.W. 1961. Competition between bacterial strains affecting nodulation in soybeans. *Proc. Soil Sci. Soc. Am.* **25:** 105–108.

Montange, D., Warembourg, F.R., and Bardin, R. 1981. Utilisation du $^{15}N_2$ pour estimer la fixation d'azote et sa répartition chez les légumineuses. *Plant Soil* **63:** 131–139.

Munevar, F. and Wollum, A.G. 1981. Growth of *Rhizobium japonicum* strains at temperatures above 27°C. *Appl. Environ. Microbiol.* **42:** 272–276.

Phillips, D.A. and Teuber, L.R. 1985. Genetic improvement of symbiotic nitrogen fixation in legumes. *Nitrogen Fixation Research Progress Abstracts* 1985: 11–17.

Schmidt, E.L. 1974. Quantitative autoecological study of microorganisms in soil by immunofluorescence. *Soil Sci.* **118:** 141–149.

Trinick, M.J. 1982. Biology. In: *Nitrogen Fixation.* Vol. 2. *Rhizobium.* W.J. Broughton, ed. Oxford Science Publications, Oxford, pp. 76–135.

Vincent, J.M. 1970. *A manual for the practical study of root-nodule bacteria.* IBP Handbook no. 15. Blackwell Scientific Publications, Oxford.

Vincent, J. 1974. In: *The Biology of Nitrogen Fixation.* A. Quispel, ed. North Holland, Amsterdam, pp. 265–341.

Wacek, T.J. and Brill, W.J. 1976. Simple, rapid assay for screening Nitrogen-fixing ability in soybean. *Crop Science* **16:** 519–523.