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# SOIL MICROBIAL ECOLOGY

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## Rhizobial Ecology and Technology

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### I. INTRODUCTION

Broadly defined, *biotechnology* encompasses all technology based on biological processes and products. As such, fermentation-based inoculant production and application practices can be said to be 100-year-old agricultural biotechnologies (Quispel, 1988). The commercial value of bacteria forming N<sub>2</sub>-fixing symbioses with leguminous plants was recognized shortly after its discovery more than a century ago. Patents were issued before the turn of the century covering the bacterial product.

In the United States, Canada, Australia, and other industrial countries, demonstration of benefits from inoculating certain legumes with rhizobia has led to the formation of commercial enterprises based on inoculant production technology. However, the bulk of our knowledge and research effort on biological N<sub>2</sub> fixation occurred during and after the 1970s in response to shortages of oil. As the price of fossil fuel-derived N fertilizer increased, so did the demand for biological alternatives. This was particularly so in developing countries, where both local supplies and the hard currency for purchasing fertilizers on the international market were in short supply.

On a global scale, N<sub>2</sub>-fixing legumes are the major source of N to the biosphere, although estimates of the actual amounts vary considerably (Evans and Barber, 1977). In developing countries, legumes are often an integral part of forest, pastures, and agricultural ecosystems. To break the hold of poverty, keep pace with population growth, and improve the standard of living, farmers in developing countries must increase food and fiber production, without a concomitantly significant increase in inputs. The high-input agricultural practices of the more industrialized nations of temperate zones are rarely suitable for tropical conditions in most developing countries (see Chap. 20). Biological processes capable of improving agricultural productivity, while minimizing soil loss and ameliorating adverse edaphic conditions, are essential.

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An important consideration for optimizing N<sub>2</sub> fixation in the legume–rhizobial symbiosis is the response of the microsymbiont and the nodule to a dynamic soil environment. A better understanding of rhizobial ecology is crucial to any successful application and acceptance of inoculant technologies for improving and sustaining agricultural ecosystems. Throughout this chapter the use of terms rhizobia or rhizobial refer to bacteria of the genera *Rhizobium* and *Bradyrhizobium*, unless otherwise specified.

## II. RHIZOBIAL ECOLOGY IN RELATION TO APPLIED BIOLOGICAL NITROGEN FIXATION

In the absence of the host, rhizobia are but ordinary soil bacteria, with any genetic or ecological advantage realized only in the presence of a host root. As free-living members of the soil microflora, rhizobia are subject to prevailing physiochemical and biological conditions and are forced to compete with other organisms for limited resources. When free-living, rhizobia are in their saprophytic phase. When presented with the root of a receptive host, rhizobia gain an advantage because they possess the ability to infect and gain entry into the root (*infective phase*) and, eventually, cooperate in the formation of a functioning root nodule (*symbiotic phase*).

An understanding of the ecological factors that regulate the response to legume inoculation can guide strain selection for new products while, at the same time, improving quantitative models for predicting the benefits and costs of legume inoculation in different situations. Comprehensive reviews on the ecology of rhizobia already exist in the literature (Bottomley, 1991; Bushby, 1982; Lowendorf, 1980; Parker et al., 1977). The following discussion is not intended as a similar comprehensive review, but rather, as an abridged discussion to highlight those biotic and abiotic factors that most affect the performance of rhizobia and thereby contribute to success or failure in the field. These factors are discussed in relation to the saprophytic, infective, and symbiotic phases of the *Rhizobium* and *Bradyrhizobium* life cycle.

### A. Saprophytic Phase

Relations between selected environmental variables and the abundance of free-living rhizobia have been examined in detail (Lawson et al., 1987; Woome et al., 1988; Yousef et al., 1987). One fundamental paradigm is the importance of the host legume and the cropping history in maintaining populations of rhizobia in soils. Host legumes are influential by means of rhizosphere effects, which to a great extent, modify the roles played by physicochemical characteristics on the distribution and population size of saprophytic rhizobia (Pena-Cabriales and Alexander, 1983).

The most problematic environments for rhizobia are marginal lands with low rainfall, extremes of temperature, acid soils of low nutrient status, and poor water-holding capacity (Bottomley, 1991). Populations of *Rhizobium* and *Bradyrhizobium* species have been shown to vary in their tolerance to these major environmental factors and, consequently, the screening for tolerant strains has been pursued.

Keyser and associates (1979) found that cowpea rhizobia, previously identified as acid- and Al-tolerant, formed more nodules in two acidic soils than did sensitive strains. Acid-tolerant inoculant strains of *R. meliloti* helped establish large areas of *Medicago polymorpha* on acidic soils in Australia (Howieson and Ewing, 1986; Howieson et al., 1988).

In contrast, Bottomley (1991) suggested that the influences of specific abiotic factors, such as organic matter or clay content, pH, base saturation, and such, which were shown by various researchers to influence saprophytic rhizobia, are much less important than their interactive effects. Also, Ozawa and co-workers (1988) showed that spatial distribution and microhabitat effects are also important, when they demonstrated a greater persistence of introduced *B. japonicum* inside soil aggregates than on aggregate surfaces.

Following introduction of exotic rhizobia into nonsterile soils, the size of the introduced population often declines rapidly. This has been demonstrated in short-term studies for several *Rhizobium* and *Bradyrhizobium* species (Bohloul and Schmidt, 1973; Boonkerd and Weaver, 1982; Pena-Cabriaes and Alexander, 1983; Vidor and Miller, 1980). Longer-term studies describe the kinetics of this decline and the attainment of a survival equilibrium (Corman et al., 1982; Crozat et al., 1982).

The relation between environment and the abundance of native rhizobia at 14 diverse sites on the island of Maui, Hawaii, was described by Woomeer and coauthors (1988). Populations varied from 1.1 to 4.8 log<sub>10</sub> cells/g of soil. The presence of a particular *Rhizobium* or *Bradyrhizobium* sp. was correlated with the occurrence of its appropriate host legume. Total rhizobial numbers were significantly correlated with mean annual rainfall, legume cover and shoot biomass, soil temperature, extractable bases, and P retention. Persistence of introduced rhizobia in these same environments showed that soil type affected survival of individual species. Significant differences were observed at each one of the sites (Woomeer, 1990).

## B. Infective Phase

There is accumulating evidence that the two symbiotic partners interact at the level of gene expression by reciprocally transmitting signals for the activation of symbiotic genes in the early stages of infection (Kondorosi et al., 1988). In contrast, our knowledge of the environmental influences on these infective stages is much less detailed. The infection process is perhaps the most sensitive phase of the life cycle to stresses of the environment, as is documented for acidity (Munns, 1977) and salinity (Singleton and Bohloul, 1984).

By using a split-root growth system, Singleton (1983) demonstrated that functional components of the soybean-bradyrhizobial symbiosis can be independently subjected to salinity stress. Nitrogenase activity of nodules was more a function of the degree of stress in the shoot than a direct effect of salt on nodular processes (Singleton and Bohloul, 1983). Initial processes involved in soybean nodulation were extremely sensitive to NaCl, much more so than nodule development (Singleton and Bohloul, 1984). Early processes related to infection in alfalfa and *Stylosanthes* species were also impaired by acidity (Carvahlo et al., 1982; Munns, 1968a).

It has long been known that the host legume exerts substantial influence over the early events of infection (Vest et al., 1973). Nutman (1948) showed that the nodule number of *Trifolium pratense* was regulated by the host and restricted to foci of meristematic activity. Bhuvanewari et al. (1980,1981) demonstrated that a host root is only transiently susceptible to rhizobial infection between the zone of elongation and the position of the smallest emergent root hair. Research by Munns (1968b) on *Medicago sativa* showed that the appearance of the first group of nodules inhibited or suppressed subsequent nodule formation.

The apparent host response that limits the development of nodules to those resulting from the earliest infections has profound implications on interstrain competition for nodule occupancy. Skrdleta (1970) demonstrated that nodulation by a second strain was reduced if it was introduced to the soybean root as soon as 13 days after a previous inoculation. Subsequent research has shown that even a preexposure period as short as six hr can completely change nodule occupancy patterns (Bohlool et al., 1984; Kosslak et al., 1983). Singleton (1983) reported that when one-half of a split-root system was inoculated 48 hr before inoculation of the other, nodulation on the second side was significantly reduced. Kosslak and Bohlool (1984) showed how this phenomenon was related to the photosynthetic potential of the plant. Postinfection control of nodule development to optimize fixation was reported by Singleton and Stockinger (1983). They showed that the host could compensate for ineffective nodulation by increasing the mass of effective nodule tissue. In split-root experiments,  $^{14}\text{CO}_2$  tracers were used to demonstrate that the host preferentially allocated current photosynthate to localized areas of N assimilation (Singleton and Van Kessel, 1987).

### C. Symbiotic Phase

Rhizobial populations in a soil show heterogeneous effectiveness at reducing  $\text{N}_2$  in symbiosis with a particular host (Brockwell et al., 1988; Singleton and Tavares, 1986), competitiveness for nodule formation (Hardarson and Jones, 1979; Materon and Vincent, 1980; May and Bohlool, 1983), and various physiological characteristics (Bezdicsek, 1972; Zablutowicz and Focht, 1981). Although rhizobia are competent saprophytes and rhizosphere organisms, their population densities are seldom high when legumes are not a large component of the plant community (Bohlool and Schmidt, 1983; Kucey, 1989; Woomer et al., 1988). These observations suggest that the symbiotic state is critical to the establishment of a substantial saprophytic population of rhizobia in the soil.

The establishment and functioning of an effective symbiosis is dependent on genetic determinants in both the plant and bacterium. Some 45 genes across eight legume species have now been identified as affecting nodulation and  $\text{N}_2$  fixation (see Chap. 6; Vance et al., 1988). The establishment of rhizobia in the symbiotic state results from a competition for nodule sites among strains during the infection stage. One of the major problems in inoculation technology is the establishment of introduced strains in the nodules of legumes grown in soils that contain indigenous populations of other rhizobia (Ham, 1980; Vest et al., 1973). Previous inoculation and continued cropping of a legume confer a formidable advantage in numbers and environmental adaptation to the indigenous population in competition with the introduced strains. Recent experiments from standardized field inoculation trials at 29

sites showed that 59% of the variation in inoculation response could be accounted for by numbers of indigenous rhizobia (Thies et al., 1991).

Successful competition by inoculated rhizobia is both strain-specific and a function of relative cell densities in the soil (Hardarson et al., 1989; Materon and Vincent, 1980; May and Bohlool, 1983; Weaver and Frederick, 1974). Important genetic factors have been identified for both symbiotic partners, such as host range (Cregan et al., 1989; Sadowsky et al., 1987), mobility of rhizobia (Catlow et al., 1990; Mellor et al., 1987), and bacteriocin production (Hodgson et al., 1985; Schwinghamer and Brockwell, 1978; Triplett, 1990). The complexities involved in competition for nodulation of legumes are numerous (Dowling and Broughton, 1986). However, in the absence of indigenous bradyrhizobia, the pattern of competition between strains for nodulation of soybean was found to be a stable, selectable characteristic, independent of rhizosphere population size, N fertilizer applications, temperature, or soil type (Abaidoo et al., 1990; George et al., 1987).

It is well established that increasing levels of mineral N in the rhizosphere will inhibit nodule formation and function. Split-root experiments have shown that N inhibits soybean nodule formation by means of localized effects on the root system, rather than as a function of whole-plant nutrition (Eaglesham, 1989; Hinson, 1975). Concentrations of available N above 2 mM generally inhibit N<sub>2</sub> fixation, whereas, under controlled conditions, 15 mM N has decreased nodule number 2.5-fold (Malik et al., 1987). In the absence of indigenous rhizobia, Thies et al. (1991) found that inoculation response was directly proportional to the availability of mineral N in the soil.

## II. RHIZOBIAL TECHNOLOGY

Inoculant production is the manufacturing step by which promising strains of *Rhizobium* and *Bradyrhizobium* spp. are mass-produced and commercially formulated for use by the farmer. Decisions to develop inoculant production capability must be justified based on economic response to legume inoculation and a market demand. In the following discussion, the various aspects of the technology that relate to the process of inoculant production and use are discussed.

### A. Rhizobia for Inoculant Production

An essential first step in legume inoculant production is the selection of appropriate strains that are already in use for such. Some of the sources for pure cultures of proved effectiveness for research purposes and for inoculum production include the Microbiological Resource Centers (MIRCENs) at Nairobi (Kenya), Porto-Alegre (Brazil), Bambey (Senegal), USDA-Beltsville (Maryland), and the NifTAL Project (Hawaii). The rhizobial germplasm resource at the NifTAL Project maintains tested rhizobial strains recommended for inoculation of various grain, forage, and tree legumes.

The desirable microbial qualities of rhizobia for use in the preparation of rhizobial inoculants described by Brockwell et al. (1982), Burton (1979), Date (1976,1982), and Walter and Paau (1992) are summarized as follows:

1. Ability to induce nodule formation and N<sub>2</sub> fixation on the target legume or legumes

2. Ability to infect and produce nodules in the presence of competing rhizobia populations
3. N<sub>2</sub>-fixing ability over a range of environmental conditions
4. Nodule formation and N<sub>2</sub> fixation in the presence of soil N
5. Good growth in media, in the carrier material, and in soil
6. Persistence in soil, particularly for reseeded annual legumes
7. Ability to migrate from the initial site of inoculation
8. Ability to colonize soil in the absence of host root influence
9. Ability to tolerate environmental stresses
10. Ability to support N<sub>2</sub> fixation in association with a wide range of host genotypes or cultivars
11. Genetic stability during storage
12. Compatibility with agricultural chemicals and other seed dressings

## B. Fermentation Systems

### *Media for Mass Culture*

Commercial inoculant production requires the cultivation of large volumes of rhizobia. The ingredients that are used in the formulation of the liquid culture media must contribute to the overall cost efficiency of inoculant production. Several media formulations were listed by Burton (1979). Various industrial by-products have also received attention for the mass culture of rhizobia. Corn steep liquor (Burton, 1979) and proteolyzed pea husks (Gulati, 1979) have been used as a C source and for providing growth factors for various rhizobial species. Malt extract has yielded cell densities in excess of  $5 \times 10^9$ /ml in mass culture of *R. leguminosarum* bv *phaseoli* and *R. leguminosarum* bv *viceae*, *R. meliloti*, and *B. japonicum* (Bioardi and Ertola, 1985). In unsupplemented whey, a by-product of the cheese industry, the *R. meliloti* viable cell density reached  $5 \times 10^9$ /ml in 48 hr of incubation, and in whey supplemented with yeast extract (1.0 g/L) and phosphate (0.5 g/L), viable densities reached  $10^{10}$ /ml (Bissonnette et al., 1986). Viable cell counts of *R. leguminosarum* bv *viceae* in a 200-L-capacity industrial fermentor were 100-fold higher in a medium containing yeast extract (5 g/L), as the sole C and N source, compared with the cell multiplication in the standard mannitol-yeast extract medium (Meade et al., 1985). Undefined media constituents can have significant effects on product performance. For example, the yeast extract concentration can be critical, as it was shown that in two strains of *R. meliloti*, the N<sub>2</sub> fixing or nodulating ability, or both, were either impaired or lost during cultivation on media containing > 1.0% yeast extract (Staphorst and Strijdom, 1972).

### *Fermentors for Mass Culture*

In contrast with industrial microorganisms that are cultured for their by-products or constituents, the mass production of rhizobia is aimed at producing high numbers of viable cells in a short time. However, like other industrial microorganisms, rhizobia are cultured aseptically in fermentors. The culture medium, rhizobial strain, temperature, agitation, pH, and aeration are the main factors affecting population growth and density that can be controlled. With a good culture medium and adequate aeration, cell densities of 4 or  $5 \times 10^9$ /ml can be obtained in 96 hr with a 1.0% inoculum (Burton, 1979), and viable densities ranging from  $1.5$  to  $2.3 \times 10^{10}$  cells/ml

are possible in small-volume fermentors under certain experimental conditions (Balatti et al., 1987). Further treatment of fermentor capacity and design for production of rhizobial biomass can be found in Roughley (1970), Smith (1987), Somasegaran and Hoben (1985), and Thompson (1980).

In most developing countries, acquisition of suitably sized, affordable fermentors and the level of skilled expertise for their operation presents problems for establishing inoculant production capability (Persuad, 1977). The NifTAL Project has been involved in the development of low-cost, simple-to-operate fermentor designs for conditions in developing countries. One suitable design that has been under evaluation at NifTAL since 1984 is shown in Figure 1. The NifTAL-designed fermentor is a stainless steel pressure vessel with a total volume of 141 L. The vessel serves first to sterilize the culture medium and, subsequently, for cell multiplication. Initial sterilization of the medium is achieved by heating with a 98,000 BTU four-ring gas burner. In evaluation trials, 100 L of culture medium were sterilized per run and viable cell counts  $> 2.5 \times 10^9$  cells/ml were obtained during fermentation.

Another fermentor design (UPIL, Unité de Production d'Inoculum pour Légumineuses) has been tested in Rwanda (Macary et al., 1986). Unlike the NifTAL-designed fermentor, UPIL has a 30 L capacity and is sterilized by placing the whole fermentor in a suitable autoclave. The temperature control during growth of the culture is achieved by placing the whole fermentor in a water-bath.

### C. Inoculant Formulations

The first rhizobial inoculants were pure cultures propagated on solid medium (agar slants) and were marketed in 8- to 10-oz (240–300 ml) glass bottles (Fred et al., 1932). Since then, the methods of producing inoculants for commercial application have undergone notable evolution to meet present-day market demands. Most inoculants produced today consist of an absorbant solid carrier material that is blended with liquid rhizobial cultures. The use of a carrier greatly facilitates the production and product application. Other formulations, which are not currently of widespread use with rhizobia, are discussed in Chapter 21.

#### *Carriers*

The properties that must be considered in producing a good carrier material include (1) high water-holding capacity, (2) toxicity to rhizobia, (3) ease of sterilization by autoclaving or gamma-irradiation, (4) material availability and price, (5) adhesive properties for coating seeds, and (6) pH-buffering capacity and high cation- or anion-exchange capacity (*BNF Bulletin*, 1991; Burton, 1981). The most frequently used carrier material for inoculant production is peat, an extremely complex material highly variable in its physical, chemical, and biological properties. The choice of peat as the preferred carrier for rhizobia is supported by numerous studies in which it was established that the peat provided better protection for the rhizobia in the package and on the inoculated seed. Rhizobia can grow in peat without nutrient supplements, as evident from the multiplication and survival when added as diluted cultures (Somasegaran, 1985; Somasegaran and Halliday, 1982).

Chemical and physical analysis of a commercial peat is shown in Table 1. Importantly, chemical analysis of a particular peat may not always reflect its quality as a suitable carrier. There are many peat deposits throughout the world, but only

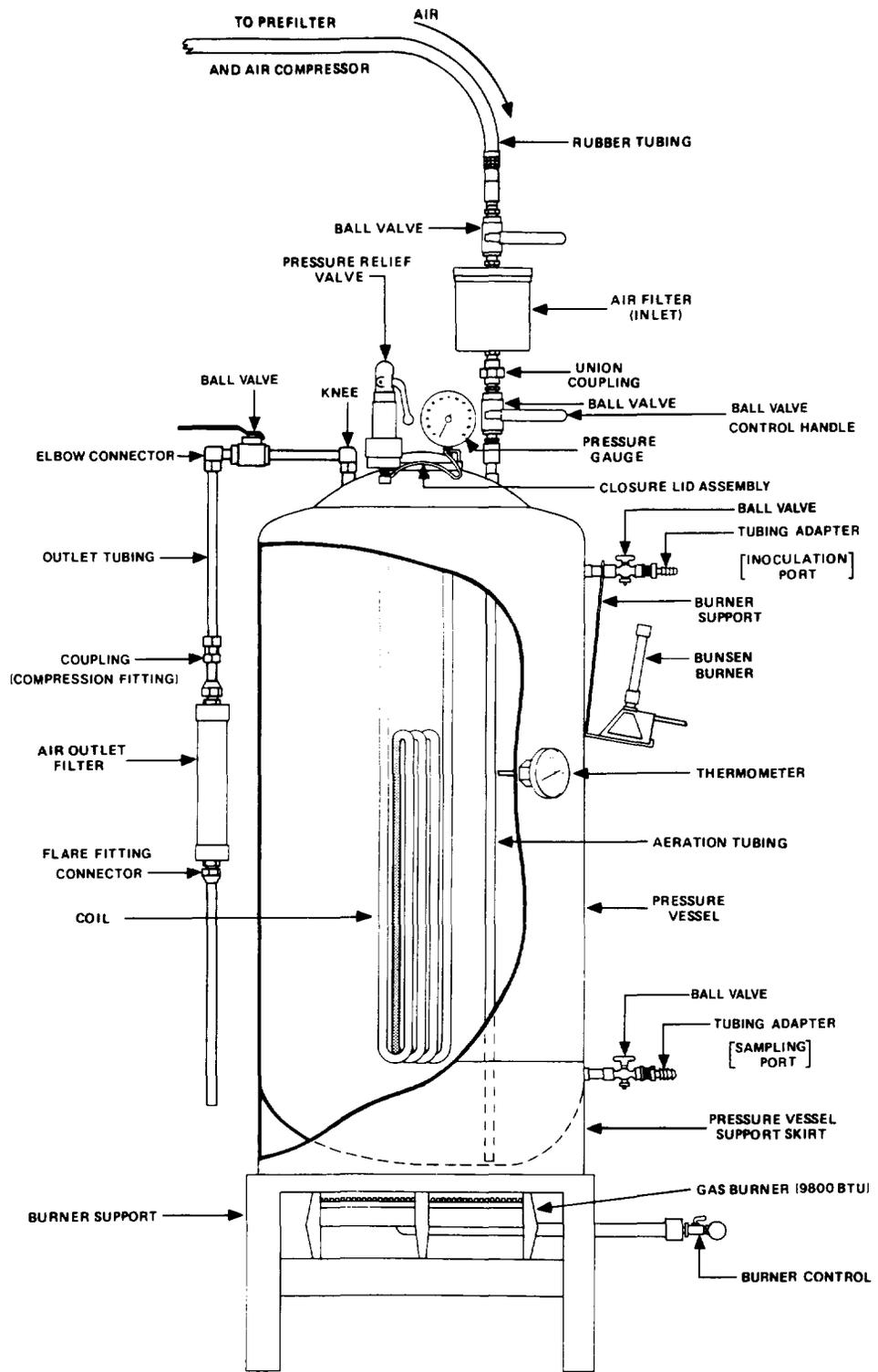


Figure 1 The NifTAL-designed fermentor, 141-L volume.

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**Table 1** Characteristics of Sedge Peat Used for Commercial Inoculant Production in the United States

Sedge peat contents	Amounts
Total nitrogen (%)	1.62
Organic matter (%)	86.8
Ash (500°) (%)	13.2
Exchangeable potassium (ppm)	62
Nitrogen as NH <sub>4</sub> and NO <sub>3</sub> (ppm)	94
Available phosphorus (ppm)	12
pH	4.5–5.0
Moisture (%)	7–8
Analysis of ash (%)	
Potassium	1.12
Phosphorus	0.33
Calcium	5.21
Magnesium	1.14
Iron	2.10
Silicon	28.0
Aluminum	6.32
Sodium	0.52

Source: Burton (1979).

about one of out nine proves satisfactory for survival of rhizobia for use as a carrier (Graham et al., 1974). The choice of a peat acceptable for inoculant production can be made only on actual tests of its suitability for growth and survival of rhizobia (Roughley and Vincent, 1967).

Because suitable peats are not always available in certain countries, other carrier materials have been evaluated for use in inoculum production. The varieties of carrier materials that have been evaluated for production are listed in Table 2. In almost every case, the various carrier materials have been amended with nutrients to support growth and survival of the rhizobia. For example, mixtures that have been tested are as diverse as low-grade Indian peat and a farmyard manure-tank silt mixture, (Bajpai et al., 1978), soil plus coir dust or soybean meal (Isawaran, 1972; John, 1966), a coal–bentonite mixture supplemented with lucerne meal (Deshodt and Strijdom, 1976), and vermiculite with various nutrient supplements (Graham-Weiss et al., 1987).

Irrespective of the chosen carrier, it is important that marked carrier–rhizobial interactions cannot be avoided. Very large numbers of a strain of *R. meliloti* survived for 140 days in a corncob and soil-based mixture, whereas there was relatively poor survival of a *Bradyrhizobium* sp. and a *B. japonicum* strain in the same carrier (Deshodt and Strijdom, 1976). The growth and survival in peat of three strains of *Rhizobium* representing three cross-inoculation groups were affected by the source of peat, pH, and the methods employed for pH adjustment, sterilization, drying temperature, and the final moisture content preliminary to milling (Roughley and Vincent, 1967). The various interactions among rhizobial peat source and method of peat sterilization have been reported for peats from Mexico, Argentina, Spain, and the United States (Somasegaran, 1985).

Table 2 Suitable Carrier Materials Characterized for Inoculant Production

Carrier material	Rhizobial species	Country	Reference
Peat	<i>R. l. bv trifolii</i> , <i>R. meliloti</i> <i>Bradyrhizobium</i> spp.	Australia	Roughley and Vincent, 1967
Lignite	<i>Bradyrhizobium</i> spp.	India	Kandasamy and Prasad, 1971
Modified lignite	<i>B. japonicum</i>	India	Dube et al., 1973
Coal-bentonite-lucerne	<i>R. meliloti</i> <i>B. japonicum</i> <i>Bradyrhizobium</i> spp.	South Africa	Deschodt and Strijdom, 1976
Filter mud	<i>R. l. bv trifolii</i> , <i>Bradyrhizobium</i> sp.	Australia	Philpotts, 1976
Peaty-soil, farmyard manure	<i>R. l. bv viceae</i>	India	Bajpai et al., 1978
Coal	<i>R. l. bv trifolii</i>	Colombia	Halliday and Graham, 1978
Polyacrylamide	<i>B. japonicum</i>	West Africa	Dommergues et al., 1979
Coal	<i>R. l. bv phaseoli</i>	United States	Paczkowski and Berryhill, 1979
Charcoal-bentonite	<i>B. japonicum</i>	India	Bhatnagar et al., 1982
Vegetable oils, peat, bentonite, charcoal	<i>R. l. bv phaseoli</i>	United States	Kremer and Peterson, 1983
Mineral soils	<i>R. meliloti</i> <i>B. japonicum</i> <i>R. l. bv phaseoli</i>	United States	Chao and Alexander, 1984
Vermiculite	<i>R. meliloti</i> <i>B. japonicum</i> <i>R. l. bv viceae</i> <i>R. l. bv phaseoli</i>	United States	Graham-Weiss et al., 1987

Powdered and granular peats are the principal solid inoculants. The desired peat particle size to produce a powder is in the 60–100  $\mu\text{m}$  range, whereas a particle size of 400–1200  $\mu\text{m}$  results in a granular material. The powdered form is commonly applied to the seed immediately before planting. The inoculant is made to stick to the seed by coating its surface with a suitable adhesive material before applying the inoculant. It is not always possible to directly inoculate seeds with the powdered form, such as is the case with peanuts (*Arachis hypogaea*), and beans (*Phaseolus vulgaris*) that have been coated with pesticides. Peat-based granular inoculants for soil application have the capability of providing more rhizobia than does direct seed inoculation with powder, which is important when rhizobial population density is the limiting factor to successful inoculation. For example, in experiments involving granular and powder inoculants, higher soybean yield and better nodulation were obtained with the granular carriers (Bezdicsek et al., 1978). Granular application in the furrow avoids any direct contact between rhizobia and any chemical seed dressings and avoids mechanical disruption of delicate seed coats, such as with *A. hypogaea* (Smith, 1987).

#### Sterile Versus Nonsterile Carrier-Based Inoculants

It has been pointed out that inoculants of inferior quality can be held partly responsible for the fact that progress in adopting rhizobial technology is slow in many countries

(Strijdom and Van Rensburg, 1981). Producers in Australia (Roughley, 1970) and South Africa (Strijdom and Van Rensburg, 1981) manufacture only high-quality products, whereas the sterilization requirement has restricted production capacity for quality inoculants in developing countries. Large-scale commercial producers of inoculants in the United States use flash-dried (650°C) and ground peat for the preparation of inoculants (Burton, 1979).

Inferior quality is usually because the product contains too few viable rhizobia to ensure prompt and effective nodulation. Sterilization methods include gamma-radiation and autoclaving. Sterilization has a great influence on the growth and survival of rhizobia and their ability to achieve consistently high cell densities in excess of  $10^9/g$  (Parker and Vincent, 1981; Roughley and Thompson, 1978; Roughley and Vincent, 1967; Somasegaran, 1985; Somasegaran and Halliday, 1982; Strijdom and Van Rensburg, 1981). Because inoculants prepared with sterilized carriers are virtually pure cultures of rhizobia, enumeration of rhizobia to determine inoculant quality is easily accomplished by simple plate count methods (Roughley and Vincent, 1967; Hoben and Somasegaran, 1982). Contamination is another important factor in determining the growth and survival of desired rhizobial strains in a carrier (Van Schreven, 1970). Rhizobia are relatively slow growing and, therefore, are weakly competitive in the presence of more vigorous microbial contaminants. In one study, multiplication of various rhizobia in nonsterile peat was always less than threefold, and for pea and lupin rhizobia, the numbers actually declined. In sterilized peat, multiplication occurred at significantly higher rates (Roughley and Thompson, 1978).

#### *Single Versus Multistrain Inoculants*

Both single-strain and multistrain inoculants are available (Burton and Martinez, 1980). Multistrain products may contain rhizobial strains from two distinct cross-inoculation groups (e.g., strains for *Trifolium* and *Medicago* spp.), or a mixture of strains for a single group (Roughley and Pulsford, 1982). In Australia, only single-strain inoculants are produced. This is to prevent the potential for antagonistic effects by one particular strain and to provide better quality control (Schwinghamer and Brockwell, 1978; Thompson, 1980). In a recent study, the importance of maintaining a balance among the component strains of a multistrain inoculant was demonstrated in inoculant formulations for *Phaesolus vulgaris* (Somasegaran and Bohlool, 1990). In this study, the amount of  $N_2$  fixed by a multistrain inoculant was 51.8% lower than that of the most effective strain applied alone, whereas the most effective strain in the mix fixed  $N_2$  at rates 100-fold less than any other strain. In another study, it was shown that  $N_2$  fixation by chickpea, soybean, and dry-bean inoculated with multistrain products depended on both the effectiveness of the component strains and the percentage of the nodules they occupied (Somasegaran and Bohlool, 1990). Few other critical evaluations of single- and multistrain inoculants exist; consequently, generalizations are difficult (Thompson, 1988).

#### *Multipurpose Inoculants*

The practice of inoculating legume seeds with rhizobia immediately before planting offers the opportunity to introduce other beneficial organisms or chemicals into the rhizosphere. The success of this strategy depends on the compatibility between the rhizobial strain(s) and the chemicals or other organisms employed. The use of *Bacillus thuringiensis* as a biological insecticide is well known, and a multipurpose

inoculant consisting of rhizobia and *B. thuringiensis* warrants investigation. Other bacteria, such as *Pseudomonas fluorescens* that produce siderophores, may enhance rhizobial survival and aid in Fe nutrition. Fungi may also aid in suppressing root diseases and improve legume stands. Hume and Blair (1990) found that a strain of *Trichoderma* increased nodulation on soybean when coinoculated with *B. japonicum*, although the response was limited to cases in which the rhizobial population in the inoculant was low.

#### D. Quality Assessment

The control of product quality at the national level is difficult, especially in countries with numerous large and small manufacturers. The various approaches and techniques that have been used for assessing quality control of rhizobial inoculants in Australia were described by Thompson (1980). A control organization, the Australian Inoculant Research and Control Service, is charged with assuring product quality. Unlike Australia, inoculant manufacturers in the United States are responsible for selecting their own rhizobial strains and setting their own quality control standards (Burton, 1979). Poor quality control during the production, storage, or distribution will result in the application of poor quality inoculants.

As indicated earlier, poor product quality is an most important factor that can result in inoculation failure. Poor quality inoculants commonly fail to provide sufficient numbers of the appropriate rhizobia at the proper time for initiating nodulation. The quality of several chickpea (*Cicer arietinum*) inoculants was compared by Toomsan et al. (1984), who found major discrepancies between counts of total bacteria and total rhizobia. This is shown in Table 3.

**Table 3** Chickpea *Rhizobium* Populations in Various Peat Inoculants Estimated by Plate and Plant Infection Dilution (MPN) Methods<sup>a</sup>

Sample	Plate count		Plant infection dilution method	
	Contaminant bacteria/g peat	<i>Rhizobium</i> -like colonies/g peat	MPN count/g peat	95% fiducial limits ( $\pm$ )
1	Nil	10.02	9.94	0.44
2	Nil	9.82	9.26	0.44
3	Nil	9.48	10.26	0.62
4	Nil	9.57	9.94	0.62
5	Nil	9.66	9.94	0.62
6	8.25	8.32	7.08	0.44
7	9.63	10.26	3.40	0.44
8	9.41	9.35	7.58	0.44
9	8.26	9.01	8.57	0.62
10	9.56	8.40	5.94	0.44
11	8.63	8.03	4.24	0.62
12	8.41	8.44	7.65	0.44
13	7.59	9.25	8.58	0.62
14	7.67	8.56	5.24	0.62

<sup>a</sup>Numbers expressed as log<sub>10</sub>.  
Source: Toomsan et al. (1984).

The minimum numbers of rhizobia required to guarantee good nodulation are considered to be  $2 \times 10^7$  cells/g inoculant, or 300 cells per seed. However, numbers as high as  $10^4$ – $10^6$  rhizobia per seed are preferred, especially when there is substantial competition with native strains (Centro Internacional de Agricultura Tropical, 1988). The standard required of the sterilized peat products in Australia, Canada, and New Zealand is  $10^9$  rhizobia per gram, with less than 0.1% contamination (Thompson, 1980; Roughley, 1982). Nonsterile carriers are used almost exclusively in the United States, with the best quality inoculants containing rhizobial numbers of  $10^8$ – $10^9$ /g. The absence of quality control regulations allows some very poor quality products to be sold (Hiltbold et al., 1980).

#### *Plate Count*

Plate count methods are sufficiently reliable for assessing high-quality inoculants with higher than  $10^8$  rhizobia per gram of carrier (Date and Vincent, 1962; Vincent, 1970). A comparison of the various plate count methods for enumerating rhizobia in irradiated peat inoculants indicated that most methods were interchangeable (Hoben and Somasegaran, 1982). Inoculants prepared with nonsterile carriers contain microorganisms in addition to the rhizobial strains. Because many bacteria have colony appearances similar to those of rhizobia enumeration of nonsterile carrier products can be problematical. A completely selective medium that will allow growth of only rhizobia has yet to be found. However, stains such as Congo red can assist in the differentiation of rhizobia from other bacteria in the inoculants (Hahn, 1966). Incorporation of cycloheximide and rose bengal have also been used to partially overcome this problem (Olsen et al., 1982; Vincent, 1970).

#### *Plant Infection Technique*

Much research has been focused on improving existing techniques and developing new ones to use in establishing quality assurance guidelines. One method, the plant infection technique, is based on the ability of rhizobia to produce nodules on legume seedlings in gnotobiotic culture. Through the use of a preenriched plant infection technique, Biederbeck and Walker (1981) demonstrated the enhanced efficacy of the method. Estimates of rhizobia in alfalfa and sainfoin inoculants were seven and four times greater, respectively, than with standard plate counts. A recent improvement to this method is the use of a computer program called most-probable-number enumeration system (MPNES), a program that generates solutions and confidence limits for population estimates of MPN data (Woomer et al., 1990). The MPNES is currently being adopted as the official standard procedure for inoculant quality control in Canada (P. Olsen, personal communication).

#### *Serology*

Serological techniques have been used successfully as a quality control procedure. The enzyme-linked immunosorbent assay (ELISA) has been used as a strain-specific serological technique for the identification of *R. meliloti* in commercial alfalfa inoculants (Olsen et al., 1982). The same technique has been applied successfully for enumerating *Bradyrhizobium* spp. in a peat inoculant for peanuts (Nambiar and Anjaiah, 1985). The membrane filter immunofluorescence technique has also been evaluated for enumerating rhizobia in peat, with good results (Somasegaran, 1985). When compared with ELISA and immunofluorescence techniques, which give total counts (viable plus nonviable cells), the immunoblot proce-

ture makes a template of viable colonies on circular nitrocellulose membranes for identification. Correlation between the immunoblot analysis and the plant infection test was high ( $r=0.90$ ) for 16 commercial alfalfa inoculants (Olsen and Rice, 1989).

There is now no substitute for enumerating invasive/infective rhizobia in legume inoculants except by the plant infection method. The recent serological techniques are useful for enumerating strains, but not for predicting nodulation frequency.

### E. Packaging and Storage

The incorporation of the carrier material with the rhizobial culture constitutes the final step in product manufacture. In preparing inoculants with nonsterile carriers, such as peat, the broth culture is sprayed on the peat, adjusted to pH 6.5–6.8 with  $\text{CaCO}_3$  and to a moisture content of approximately 40%, on a wet weight basis. After mixing, the product is spread in a thin 25- to 30-cm layer in shallow trays and cured for a period of 48–72 hr at temperatures between 25° and 28°C (Burton, 1979).

The cured product is further processed by milling or screening to yield a homogeneous moist powder for packaging. The cured, milled product is packaged in polyethylene (0.038–0.076 mm in thickness) that retains moisture, yet allows sufficient gas exchange and controls contamination (Smith, 1987). Besides these properties, packaging material must be sufficiently strong to withstand handling. Package size varies from 40 g to 2.8 kg, depending on inoculant form and the manufacturer.

By another method, the broth culture is inoculated aseptically into sterile peat by puncturing the container and delivering the required volume of broth culture directly from the fermenter (Roughley, 1970). In contrast with nonsterile carriers, higher moisture levels (up to 60%) can be tolerated by rhizobia in sterilized peat (Roughley, 1970).

Studies of product storage are numerous. One of the most critical factors affecting rhizobial survival during storage is temperature. The influence of storage temperature on the survival of rhizobia is strain-specific and depends on the purity of the culture and moisture loss during storage (Somasegaran et al., 1983). Pure cultures of rhizobia in sterilized peat may be stored for six months at 4°C, followed by up to nine months on the shelf (Roughley, 1970). However, other studies with previously sterilized peat showed a significant decline in the viability of several strains of rhizobia when the inoculants were stored at 4°C (Roughley, 1982; Somasegaran, 1985). These data suggest that choice of low storage temperatures should be made on an individual product basis.

### F. Inoculant Application

The purpose of inoculation is to bring the rhizobia in the inoculant into contact with the legume radicle and seminal root system during and immediately after germination. The presence of a high rhizobial population surrounding the seed helps ensure prompt and effective nodulation. Successful inoculation can be achieved by direct inoculation of the seed or furrow immediately before sowing. Under most conditions, direct application to the seed is easiest and most effective.

*Seed*

Peat inoculants are commonly applied at the rate of four–six g/kg of seed, equivalent to 0.28–0.42 kg/ha at the normal seeding rate for cowpea or soybean (Food and Agriculture Organization, 1984). Rates up to 50 g/kg seed have been applied under experimental field conditions (Thies et al., 1991). High numbers of rhizobia on the seed can be maintained by coating the seeds with a suitable adhesive, such as 10% sucrose, 10% gum arabic, or 1% methyl cellulose (Roughley, 1982). A modification, termed seed pelleting, involves the use of gum arabic (40% w/v) or methyl cellulose (1.5% w/v) as adhesives, together with finely ground calcium carbonate or rock phosphate (Roughley, 1982).

*Soil*

When seeds are coated with incompatible chemicals, it is necessary that rhizobial inoculants be placed in the furrow. Inoculating the soil allows higher rates to be achieved than with seed application, ranging from 6 to 20 kg inoculant per hectare, or 1 g/m of row (Food and Agriculture Organization, 1984; Centro Internacional de Agricultura Tropical, 1988). Soil inoculation may be the more efficient method when effective rhizobia are completely lacking at the site, when conditions are hot and dry at the time of planting, in acidic soil, or where ineffective indigenous rhizobia are present (Bezdicek et al., 1978; Graham et al., 1980).

*Seedling*

The importance of leguminous trees in sustainable agriculture and forestry has fostered research to identify and evaluate methods for inoculating tree seedlings (see Chap. 9). Inoculation of seedlings in the greenhouse avoids complications encountered in the field, including competition from indigenous, less effective rhizobia. Also, a seedling nodulated by a highly effective strain may itself have a competitive advantage. Dommergues (1987) recommended spraying or drilling the inoculum directly into the container at the time of seeding or transplanting. Roskoski and associates (1986) planted peat-inoculated seeds of *Leucaena leucocephala* and *Acacia pennatula* in dibble tubes. At NifTAL, we have obtained good nodulation in several kinds of tree seedling growth containers in the greenhouse by applying rhizobial broth or peat inoculants suspended in water.

**IV. SUMMARY**

Several biotic and abiotic factors affect the performance of rhizobia in their saprophytic, infective, and symbiotic stages. Major influences are exerted by the host legume, the soil acidity, inorganic nutrients, temperature and desiccation, and competition for nodule occupancy. Rhizobial population density is correlated with the occurrence of its particular host legume, and inoculation response is strongly influenced by indigenous rhizobia and the level of available N in the soil. Significant progress has been made in the search for newer methods of strain selection, more effective and less expensive mass culture, affordable fermentors, cost-effective inoculant production systems, and improvements in quality control procedures.

## DEDICATION AND ACKNOWLEDGMENTS

Dr. B. Ben Bohlool died while this chapter was being prepared. He was committed to advancing knowledge and understanding of rhizobial ecology throughout his research career. He and his students established many important findings in this field. As Director of the NifTAL Project, he promoted and facilitated the transfer of rhizobial technology to developing countries where the practical application of biological N<sub>2</sub> fixation knowledge could be put to work. The coauthors dedicate this chapter to the memory of our colleague, Ben Bohlool. The authors appreciate the ongoing encouragement from Blaine Metting. The assistance of Susan Hiraoka, Barbara J. Sedano-Parrish, and Debra Hughes is gratefully acknowledged.

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