Chapter 11

INOCULANT PREPARATION, PRODUCTION AND APPLICATION

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1. INTRODUCTION

Progressive chemical and physical degradation of soil is a major factor affecting crop yield worldwide (Cassman, 1999). The situation is most serious in tropical regions, where soils are often structurally fragile, have low organic matter and nutrient content, and are frequently subject to erosion or inappropriate farm management. In these areas, nutrient depletion may be accentuated by the high cost of fertilizers, especially fixed-N sources, the majority of which are imported from developed countries (Hungria and Vargas, 2000; Giller, 2001). Thus, smallholders in Africa, for example, commonly apply less N, P, and K than is removed in the grain (Giller and Cadisch, 1995; Franzluebbers *et al*., 1998; Sanchez, 2002), suggesting annual average depletion rates across 37 African countries of 22 kg N, 2.5 kg P and 15 kg K ha^{-1}. Because of such depletion, biological nitrogen (N_2) fixation is critical to the agricultural sustainability of these areas, but is often constrained by the absence in the soil of efficient and competitive rhizobia. There is an obvious need to improve the availability, quality, and delivery of such rhizobia for every cropped legume.

The practice of transferring soil from a field where legumes have been grown to new areas being planted to the same crop, dates back to ancient times. It became the recommended method of inoculation after Hellriegel´s report on the N nutrition of

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leguminous plants in 1886, and was followed soon thereafter by the first use of rhizobial inoculants (Voelcker, 1896; Fred *et al.*, 1932). However, after over a century of rhizobial inoculation, most of the inoculants produced in the world are still of relatively poor quality (FAO, 1991; Olsen *et al.*, 1994; 1996; Brockwell and Bottomley 1995; Lupwayi *et al.*, 2000; Stephens and Rask, 2000). In this chapter, we discuss some aspects related to inoculant production and inoculation. Complementary information can be obtained from other reviews (Smith, 1992; Brockwell and Bottomley, 1995; Brockwell *et al.*, 1995; Lupwayi *et al.*, 2000; Stephens and Rask, 2000; Catroux *et al.*, 2001; Date, 2001).

2. STRAIN SELECTION

Successful inoculation starts with the establishment of long-term programs of strain selection and the identification of elite strains for each legume host of interest. Emphasis in this selection program should be given to a high capacity for N_2 fixation with all commonly used cultivars of the legume in question, competitiveness with indigenous or naturalized rhizobia, tolerance to environmental constraints, and the ability to persist in soil. Selection for specific ecosystems, unusual soil physical or chemical constraints, specific environmental concerns (temperature or soil acidity), or specific local cultivars may also be important (*e.g.*, Jones and Hardarson, 1979; Hungria and Bohrer, 2000; Hungria and Vargas, 2000; Mpeperecki *et al.*, 2000; Stephens and Rask, 2000; Chen *et al.*, 2002; Mostasso *et al.*, 2002). Important characteristics not often considered in strain selection are performance in storage and culture (Balatti and Freire, 1996), genetic stability (FAO, 1991), and the ability to survive on seeds (Lowther and Patrick, 1995). These traits have also been considered by Burton (1981), Roughley (1970) and Keyser *et al.* (1993).

2.1. A Successful Approach: The Brazilian Strain Selection Program for Soybean and Common Bean

Soybean (*Glycine max* L. Merr.) was introduced to Brazil in 1882, with large-scale cultivation of bred cultivars initiated in the 1960s (Vargas and Hungria, 1997; Hungria and Bohrer, 2000). As Brazilian soils were originally devoid of bradyrhizobia that were effective with soybean (Vargas and Suhet, 1980; Hungria and Vargas, 2000; Ferreira and Hungria, 2002), inoculants were also introduced in the early 1960s, mainly from the United States. Strain selection both for locally adapted cultivars and for tolerance to the often acid-soils conditions started immediately (Döbereiner *et al.*, 1970; Peres and Vidor, 1980; Vargas *et al.*, 1992; Peres *et al.*, 1993; Hungria and Vargas, 2000) with outstanding results. However, as the national mean yield for soybean has increased from 1,166 kg ha^{-1} in 1968/69 to 2,765 kg ha^{-1} in 2002/2003, plant demand for N has also increased. Further, more than 90% of the areas cropped to soybean today have been previously inoculated and have established bradyrhizobial populations of at least 10^3 cells g^{-1} of soil. Both situations contribute to a need for more efficient and competitive strains (Hungria *et al.*, 2001b; 2002).

Strain-selection programs in Brazil initially emphasized elite strains from foreign countries, but have since changed to selection amongst adapted strains obtained from locally-grown soybeans several years after their introduction. Grain yield has always been the major factor considered, but other parameters used in the identification of superior strains have included plant vigor, N_2 (C_2H_2) reduction, total N accumulated in tissues, N harvest index, ureide content in tissues, and nodule occupancy (Peres and Vidor, 1980; Peres *et al.*, 1984; 1993; Neves *et al.*, 1985; Vargas *et al.*, 1992; Hungria *et al.*, 1998; Santos *et al.*, 1999; Hungria and Vargas, 2000). The four strains used in the production of commercial inoculants in Brazil today can each fulfill the crop´s need for N at yields greater than 4,000 kg ha^{-1} (Vargas *et al.*, 1992; Peres *et al.*, 1993; Vargas and Hungria, 1997; Hungria *et al.*, 2001b). This program continues (see Table 1) with soybean bradyrhizobia having both a higher capacity for N_2 fixation and improved competitiveness already available and soon to be released for commercial purposes.

Table 1. Nodulation, nodule occupancy, and yield of soybean cultivar BR 133 inoculated with parental and variant Bradyrhizobium japonicum strains. Experiments performed in oxisols of Londrina, State of Paraná, Brazil[1].

Treatment	Nodulation (mg pl^{-1})	Nodule occupancy by inoculated strain (% before/% after)	Increase in nodule occupancy (%)	Yield (kg ha^{-1})
C - N[2]	97 b[3]	-	-	1,928 c[3]
C + N[2]	13 c	-	-	3,444 a
SEMIA 566	134 a	23/59	156	2,723 b
Variant of 566	161 a	23/65	183	3,415 a
CB1809 (=SEMIA 586)	99 b	8/22	175	3,029 b
CB1809 variant	155 a	8/45	462	3,772 a

[1]After M. Hungria and R.J. Campo (unpublished).
[2]Non-inoculated control (C) without or with N-fertilizer (200 kg of N ha^{-1}, as urea, split twice - at sowing and at flowering time).
[3]Means of three field trials, performed in three crop seasons, each with six replicates. Within a column, values followed by the same letter are not statistically different (Duncan, $p \leq 0.05$).

As with soybean, Brazil is also the largest producer of common bean (*Phaseolus vulgaris* L.) in the world, with beans being the most important source of protein in the Brasilian diet. Average bean yields in Brazil have been very low, ca. 728 kg ha^{-1} in 2002/2003, mainly because of the limited technology used by small farmers. Lack of response to inoculation in this crop has been attributed to high populations of indigenous but ineffective common-bean rhizobia in soil (Graham, 1981; Buttery *et al.*, 1987; Ramos and Boddey, 1987; Hardarson, 1993), but soil temperature and acidity are also important in Brazil. As with soybean, the strain-selection program with beans has emphasized the isolation and selection of efficient strains from local bean soils.

Many of these strains belong to the species *Rhizobium tropici,* an organism not normally associated with beans in the centers of origin of this crop. Recently, this approach allowed the identification of the efficient, competitive and high-temperature tolerant *R. tropici* strain, PRF 81 (= SEMIA 4080). In field trials in soils already containing 10^4-10^5 bean rhizobia g^{-1}, inoculation with this strain increased both nodulation and N_2 fixation, and improved grain yield up to 900 kg ha^{-1}. PRF 81 has been recommended for use in commercial inoculants since 1998 (Hungria *et al.*, 2000a) and promotion of inoculation through active extension programs has increased by 25% the sale of bean inoculant in a three-year period. The search within local indigenous populations continues, with two other *R. tropici* strains from the Cerrado area (H12 and H20; Mostasso *et al*., 2002; Hungria *et al*., 2003) also contributing to significant yield increases in beans (Figure 1). Searching for strains within a naturalized population is a time-consuming process involving thousands of plates, and many greenhouse and field trials, but a further advantage is that the strains obtained are not genetically modified, avoiding legal and socioeconomic problems.

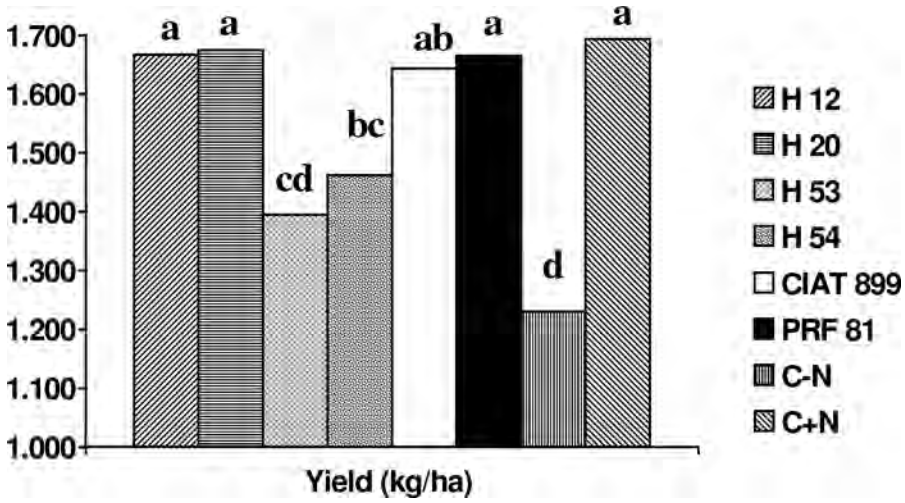

Figure 1. Effects of inoculation with Rhizobium tropici strains on yield of common bean. Uninoculated control treatments received either no N or 60 kg of N ha^{-1}, split between sowing and flowering. Mean of six field experiments performed in oxisols of Londrina, State of Paraná, Brazil, each with six replicates. Values followed by the same letter are not significantly different (Duncan, $p \leq 0.05$). After Hungria et al. *(2003).*

Because of the importance of soybean to the Brazilian economy and of beans to the country's nutrition, research on N_2 fixation in these crops has been well supported. As a result, the eleven inoculant manufacturers in Brazil sold a total of 14 million doses of inoculant for bean and soybean in 2001/2002, an increase of 16% over the previous two years. The situation is similar in Argentina where 10.3 million doses of inoculant were sold in 2001/2002. Unfortunately the financial

support for strain selection and extension activities with other important legume crops is low. Although an enormous effort has been made by some government institutions, with more than 150 strains now recommended for the 90+ legume species used in grain crop or green manure production, pastures, and agroforestry (Hungria and Araujo, 1995), inoculants for these species still represent less than 1% of the overall market.

The favorable situation for both soybean and bean inoculation in Brazil differs from that in many other regions of the world. Brockwell and Bottomley (1995) suggest that 90% of all inoculants used provide no practical benefits. Furthermore, Karanja *et al.* (2000) note declining inoculant production in several regions of Africa, with only 12% of farmers using inoculants. Both poor inoculant quality and extension, in those areas of the world where inoculation is ineffective or little used, need to be addressed (Hall and Clark, 1995; Marufu *et al*., 1995).

2.2. Selection of Fast-growing Strains for Soybean: Differences among Ecosystems

Fast-growing rhizobia that were able to effectively nodulate soybean were first isolated in 1982 from soils and nodules from the People´s Republic of China, which is the center of origin and diversity of this legume (Keyser *et al*., 1982). Today, they are classified as *Sinorhizobium fredii* (Scholla and Elkan, 1984) and *S. xinjiangensis* (Chen *et al.*, 1988). Fast-growing strains belonging to other rhizobial species have also been isolated from nodulated soybean in Brazil and Paraguay (Chen *et al.*, 2000; 2002; Hungria *et al.*, 2001a; 2001c). Initially, it seemed that these fast-growing soybean rhizobia were only able to nodulate unimproved genotypes (Keyser *et al*., 1982), but several modern soybean cultivars have now been reported as effectively nodulated by these strains (Balatti and Pueppke, 1992; Chueire and Hungria, 1997), which raises the possibility of their use in inoculants.

Among the advantages of using fast-growing strains are a shorter time for production of inoculant, a lower probability of contamination during the industrial process, easier establishment in the soil, and easier manipulation of genes (Chatterjee *et al.*, 1990; Cregan and Keyser, 1988). However, although high rates of N_2 fixation have been achieved in single inoculation experiments, fast-growing strains have proved to lack competitiveness against *Bradyrhizobium* isolates (McLoughlin *et al.*, 1985; Cregan and Keyser, 1988; Chueire and Hungria, 1997; Hungria *et al.*, 2001a). This limited competitiveness appears to be a function of low soil pH (Hungria *et al.*, 2001a). Buendia-Claveria *et al.* (1994) reported greater success with *S. fredii* as a soybean inoculant under alkaline soil conditions in Spain. This result reinforces the importance of strain selection under local conditions.

2.3. Use of Strains in Commercial Inoculants

In many countries, including the United States, microorganisms can be patented, with interpretation of the law often covering both artificially modified and "purified or isolated" preparations of newly discovered naturally occurring microbes. For natural microorganisms, a limitation can be that patents cover only a specific strain

and its derivatives, but a broader protection may be possible for genetic modified organisms (Keyser *et al*., 1993). Clearly, without strong patent protection, companies will not invest in strain selection and product development. A problem in countries, such as Brazil and Mexico, is that only genetically modified organisms can be patented, but these have problems in obtaining permission for field release.

The first North-American patent for pure cultures of rhizobia, and artificial inoculation, was obtained in 1896 by Nobbe and Hiltner and covered pure cultures of the desired *Rhizobium*, which were grown in flat glass bottles containing only a small amount of gelatin medium (Smith, 1992). Today, genetically modified and patented strains include a USDA *B. japonicum* strain, which is claimed to increase yield by 5-7%, and *S. meliloti* strain RMBPC-2, which is modified for NifA expression; both are commercialized by Urbana Laboratories (2002). Improvements in our knowledge of rhizobial genetics increase the potential for obtaining genetically modified rhizobia with superior symbiotic performance (Maier and Triplett, 1996; Sessitsch *et al*., 2002). Additional genetically modified strains are likely to be released soon as products containing such rhizobia have been approved for field trials in several countries. The patenting of inoculant strains can only reduce the comparative testing of different inoculant-quality rhizobia.

Countries also differ in their policies concerning recommendation of strains for commercial inoculants. In countries such as the United States, each company determines which strains will be used in their products. In other countries, such as those belonging to Mercosur (Brazil, Argentina, Paraguay and Uruguay), commercial inoculants must contain the strains recommended by an official committee of rhizobiologists.

Commercial inoculants may contain one or more strains. Multistrain products may be important and recommended for several different hosts, *e.g*., for both clover (*Trifolium* spp.) and alfalfa (*Medicago sativa*) (Roughley, 1970; Keyser *et al*., 1993), or for African acacias (Sutherland *et al*., 2000), but they may also be used for a single host (Roughley, 1970; Keyser *et al*., 1993). Strains for such mixed inoculants should be grown in separate fermentors before being mixed into the carrier. Even then, it is difficult to ensure either a balanced growth among the strains in the inoculant (Roughley, 1970; Frankenberg *et al*., 1995) or that strains perform similarly in terms of nodule occupancy. This situation probably explains why the N_2-fixation rates achieved with multistrain inoculants are often lower than those achieved with the single most effective strain (Bailey, 1988; Somasegaran and Bohlool, 1990). Benefits of single-strain inoculants would include avoidance of antagonistic effects between strains in the mixed inoculant, easier diagnosis of loss of effectiveness, and greater facility in quality control (Thompson, 1980). Today, the tendency is to use single-strain inoculants in countries with strong inoculant quality-control programs as well as in those with a tendency to recommend specific strains for each ecosystem. These countries include Australia, France, New Zealand, South Africa, and Uruguay (Date, 2001). Multiple-strain inoculants can pose special problems for legume species of lesser importance, where the manufacturer may not be able to justify economically the testing of strains in the mixture on a regular basis.

2.4. Persistence of the Strains on the Soils

Several studies have followed the persistence of exotic rhizobia introduced into sterile or non-sterile soil. In some studies, population numbers decline rapidly at a rate that varies with the environmental conditions, soil characteristics, or rhizobial strain used, among others (Gibson *et al.*, 1976; Keyser *et al.*, 1993). In other studies, the introduced inoculant strains still dominate in soil 5-15 years after introduction (Diatloff, 1977; Brunel *et al.*, 1988: Lindström *et al.*, 1990; see also Figure 2).

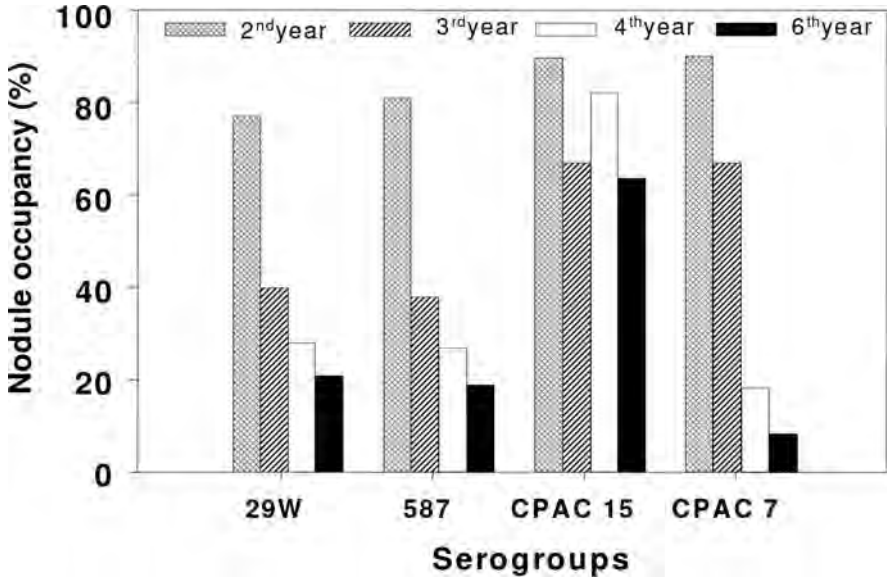

Figure 2. Dynamics of nodule occupancy by four Bradyrhizobium japonicum/B. elkanii strains for six years after their introduction into a Cerrados oxisol originally void of soybean bradyrhizobia.
Data represent the mean values of four replicates. Modified from Mendes et al. *(2000).*

Saprophytic capacity is a desirable feature of inoculant rhizobia when one is sure of both their superior N_2-fixation capacity and their genetic stability. Replacing persistent strains with more efficient ones can be difficult as is evident from numerous studies with *B. japonicum* USDA 123 in the USA (Ham *et al.*, 1971). Figure 2 shows differences in the establishment of four soybean bradyrhizobia from Brazilian commercial inoculants in a Cerrados oxisol. In this experiment, the displacement of CPAC 15 (= SEMIA 5079 and belonging to the same serogroup as USDA 123) by other strains required annual and massive reinoculation.

Similar data for the USA is provided by Dunigan *et al.* (1984). van Elsas and Heijnen (1990) have suggested that the ideal situation would be one in which the inoculant organisms could be eliminated from the environment after completing

their task. However, in some countries, the need for annual reinoculation might then significantly increase production costs. The molecular tools available today should be used to better follow the introduction, movement, and persistence of inoculant strains, and to determine the factors most important for strain persistence.

3. INOCULANT PRODUCTION

Inoculant production involves choosing and processing the carrier, culture maintenance and growth at increasing scales of production, and a final product of good quality, with a profitable benefit/cost ratio.

3.1. The Carrier

Desirable properties of a good inoculant carrier have been listed before (Keyser *et al.*, 1993; Walter and Paau, 1993; Balatti and Freire, 1996; Stephens and Rask, 2000), but can be summarized as: (i) readily available, uniform in composition and cheap in price; (ii) non-toxic to rhizobia; (iii) high water-retaining capacity; (iv) easily sterilized; (v) readily corrected to a final pH of 6.5 to 7.3; (vi) permitting good initial growth of the target organism; and (vii) maintaining high cell numbers during storage.

Peat has been the most suitable carrier for inoculant production because it usually meets these requirements. Another possible advantage of peat as a carrier is its adsorbent properties, which could reduce the effect of toxins that are built up during growth in fermentors and also lower the impact of bradyoxetin, which is a non-homoserine lactone signal molecule involved in quorum sensing that is produced by stationary-phase cultures and can inhibit *nod*-gene expression by bradyrhizobia (Loh *et al.*, 2002). Different peat sources vary in their capacity to support rhizobial multiplication and survival (Roughley and Vincent, 1967; Roughley, 1970; Somasegaran, 1985; Balatti and Freire, 1996). Among the best sources are peats from Argentina (Tierra del Fuego) and Canada, each with an organic matter content of 40-50%. For other sources, the quality of the peat can be improved by addition of humus.

If the harvested peat is wet, it should first be drained, then sieved to remove coarse material. The peat is then dried to a moisture content of about 5%, with the temperature kept below 100ºC to avoid the generation of toxic substances (Roughley and Vincent, 1967; Roughley, 1970). The peat is then ground because coarse particles adhere poorly to the seed coat. Burton (1967) and Roughley (1970) proposed that the peat be milled to pass a 0.20-0.25 mm sieve, whereas Strijdom and Deschodt (1976) recommended that 50% should pass through a 0.075 mm sieve. As many peat deposits are acid, pH should be corrected, as needed, to 6.5-7.0, usually with finely ground $CaCO_3$ (Roughley, 1970; Cattelan and Hungria, 1994).

Sterilization of the peat prior to inoculation is recommended but, unfortunately, there are still products available manufactured with non-sterile peat. Such inoculants may contain up to 1,000-fold fewer rhizobia than those made with sterilized peat, so reducing shelf life. The problem is even greater for slow-growing

rhizobia (Roughley and Vincent, 1967, Roughley, 1970; Date and Roughley, 1977; Somasegaran, 1985; Lupwayi *et al*., 2000; Stephens and Rask, 2000). Sterilization of the peat also reduces the frequency and level of contamination and, thus, the risk of introducing and disseminating plant, animal, and human pathogens (Lupwayi *et al*., 2000, Catroux *et al*., 2001). Difficulties associated with the sterilization of the peat include the identification of an appropriate, but low cost, methodology with high-capacity throughput, the need to follow aseptic procedures during culture addition to the pre-sterilized packaged carrier, and the difficulty in detecting contaminated packages (Smith, 1992; Balatti and Freire, 1996). In countries with high-quality products, such as Argentina, Australia, Canada, Czechoslovakia, France, The Netherlands, New Zealand, South Africa, and Uruguay, sterile products are either the general rule or are mandated by legislation.

For smaller quantities of peat, sterilization by autoclaving may be used with bulk peat autoclaved in either polyethylene bags or autoclave trays covered with foil at 121°C for a period of 1-3 hours (Somasegaran, 1991; Somasegaran and Hoben, 1994; Balatti and Freire, 1996). Other less-frequently used methods include fumigation with ethylene oxide or methyl bromide (Smith, 1992) and microwave radiation (Ferriss, 1984). Each of these methods has significant drawbacks. For large quantities of peat, either nuclear or γ-irradiation has been the sterilization method of choice because a more uniform final-product quality is obtained and rhizobial numbers are usually greater than obtained by autoclaving (Roughley and Vincent, 1967; Stephens and Rask, 2000). The level of γ-irradiation normally employed (5 Mrad) usually produces a sterile product (Parker and Vincent, 1981; Smith, 1992), but contaminants are consistently encountered at even higher levels (Yardin *et al*., 2000). Smith (1992) suggests that survivors from treatment at 5 Mrad do not seriously affect rhizobial growth and survival and that higher-dosage rates could result in peat toxicity as well as raising production costs (Parker and Vincent, 1981). In Brazil, where current legislation demands that dilution counts from inoculants must be devoid of contaminants at the 10^{-5} dilution, 7 million doses per year are γ-irradiated at an average cost of U$ 0.10 $dose^{-1}$, which is less than 10% of the final price; however, 7 Mrad have to be used for some peat inoculants. A more recent non-nuclear sterilization technique used in Canada uses electron beam acceleration to generate 10^7 eV, and sterilization of pre-packaged peat takes only seconds, whereas γ irradiation takes hours (Stephens and Rask, 2000).

Peat carrier is usually packaged in polyethylene or polypropylene bags, with a thickness of 0.06-0.38 mm. Bags must be resistant to sterilization, allow safe transportation of inoculant, and be readily sealable to prevent contamination. They must retain moisture, so the peat does not dry out, but allow gas exchange; both of these factors are important in retaining rhizobial viability (Roughley, 1970; Keyser *et al*., 1993). Package sizes vary considerably, generally from 40 g to 2.8 kg.

It is not always possible to use seed-applied peat as a carrier. Many soybean farmers, for instance, complain that peat-based products are time-consuming to use, especially when planting big areas. Some countries may not have natural deposits of peat, the peat available may not be suitable, or environmental regulations may

prevent its harvest because many peat reserves are located alongside rivers and streams. A number of materials with the characteristics of a good carrier have been used as alternatives with different degrees of success. These include: vegetable oils (Kremer and Peterson, 1982); mineral oils (Chao and Alexander, 1984); plant materials, such as bagasse, silk cocoon waste (Marufu *et al*., 1995; Jauhri *et al*., 1989), sawdust, rice husk (Khatri *et al.*, 1973), and corncob (McLeod and Roughley, 1961); various clays, including vermiculite (Graham-Weiss *et al.*, 1987) perlite mixed with humus (Ballati and Freire, 1996), and diatomaceous earth (Sparrow and Ham, 1983); dehydrated sludge wastewater (Rebah *et al*., 2002); polyacrylamide (Dommergues *et al.*, 1979) or cellulose gels (Jawson *et al.*, 1989); lignite and derivatives; coal; filter mud; and charcoal-bentonite (Keyser *et al*., 1993; Marufu *et al*., 1995). In contrast, survival was poor on a number of materials including diesel oil, some mineral oils, and kerosene (Faria *et al.*, 1985; Peres *et al*., 1986).

Liquid or gel-based preparations also constitute a significant percentage of the inoculant market. They are of varying composition (magnesium silicate, potassium acrylate-acrylamide, grafted starch, hydroxyethyl cellulose products) and usually include additional proprietary substances to protect the rhizobia. Several studies report good performance of these preparations when compared to peat-treated seeds (Burton and Curley, 1965; Jawson *et al*., 1989; Hynes *et al*., 1995). However, in spite of good cell numbers in plate and most probable number (MPN) counts, many of them have failed to reproduce this performance under field conditions, especially under environmental stress in Brazil (Campo and Hungria, 2000b; Hungria *et al*., 2001b; see Table 2), Uruguay and Canada.

Table 2. Soybean nodulation and yield in a Brazilian Cerrados oxisol as a result of different commercial inoculants. Experiment performed in a soil void of soybean bradyrhizobia and all inoculants contained the same strains[1].

Treatment[2]	Cells g^{-1} or ml^{-1} of inoculant	Dose	Cells $seed^{-1}$	Nodulation at flowering (mg pl^{-1})	Yield (kg ha^{-1})
C-N[3]				77 efgh	3,202 c
C+N[3]				24 h	3,334 bc
Traditional Peat Inoc.	$7.5x10^8$	500g.50kg^{-1}	$3.08x10^8$	232 a	4,226 a
Liquid 1-#1	$2.0x10^8$	400ml.50kg^{-1}	$2.80x10^3$	59 fgh	3,461 bc
Liquid 1-#2	$2.0x10^8$	800ml.50kg^{-1}	$4.95x10^4$	146 bcde	3,420 bc
Liquid 2	$1.0x10^7$	200ml.50kg^{-1}	$1.86x10^3$	43 gh	3,458 bc
Liquid 3	$1.6x10^9$	150ml.50kg^{-1}	$9.34x10^4$	133 cdef	3,363 bc
Peat 1	$2.0x10^9$	20g.50kg^{-1}	$2.80x10^4$	168 abcd	3,626 bc
Peat 2	$1.0x10^9$	200g.50kg^{-1}	$4.67x10^3$	103 defg	3,267 bc

[1]After I. C. Mendes (unpublished data). Within a column, values followed by the same letter are not statistically different (Duncan, $p \leq 0.05$).
[2]Liquid and peat inoculants are from different companies and used as recommended by manufacturers.
[3]Non-inoculated controls without or with 200 kg of N ha^{-1}, split twice - at sowing and at flowering stage.

Addition of proprietary cell protectants, sowing immediately after inoculation (Burton and Curley, 1965), and avoiding the use of fungicides with these products (Campo and Hungria, 2000a; 2000b) increases the probability of success.

Inoculants containing dried (either lyophilized or freeze-dried) and frozen (concentrated) cultures require more complex equipment for production and maintenance (Date, 2001); they are mixed with either a liquid or gel formulation at sowing (Walter and Paau, 1993). Tests performed with either dried or frozen inoculants in Brazil have shown that nodulation is usually lower than with inoculants containing bacteria prepared in the conventional way, probably because the rate of cell growth is lower than in other carriers. However, lyophilized cells may show a better survival in granular inoculants (Fouilleux *et al*., 1994). Inoculants containing polymer microcapsules, beads, or clay pellets impregnated with rhizobia, and polyacrylamide, alginate, xanthan and carob gums have also been used as inoculants (Jung *et al.*, 1982; Bashan, 1986; Smith, 1992; Walter and Paau, 1993), but survival under dry conditions is often poor (Date, 2001).

3.2. The Cultures

3.2.1. Strain Maintenance

Keeping a pure strain alive with no variation or mutation is critical to inoculant manufacture. Strains may lose desirable properties either in storage or on repeated subculture. Careful maintenance of stock cultures and periodic testing of symbiotic efficiency are essential. Lapinskas (1990) and Lupwayi *et al.* (2000) urge periodic passage through the host under field conditions to maintain symbiotic efficiency. In countries such as the United States, where strains can be patented and individual inoculant manufacturers decide which strain(s) will be used, the maintenance of cultures is mainly left to the manufacturer. Where the use of particular strains is regulated by legislation, strains are usually kept at a central facility and forwarded to the industry as needed. Several methods for short- and long-term storage have been described (see Table 3). Most long-term storage is by cryopreservation (ultra-cold conditions) or freeze-drying (lyophilization) (Vincent, 1970; Somasegaran and Hoben, 1994; Balatti and Freire, 1996; Lupwayi *et al*., 2000).

There are several sources of effective rhizobial cultures for research or inoculant production. They include the Microbial Resources Centre Network (MIRCEN), supported by the United Nations Education and Science Council (UNESCO) in Brazil (Porto Alegre), Kenya (Nairobi), Senegal (Dakar) and USA (Niftal, Hawaii (www.unesco.org/science/mircen_centres.html), and the USDA culture collection in Beltsville. The large CSIRO Tropical Pastures collection, previously maintained by Drs. D.O. Norris, R.A. Date and H.V.A. Bushby, is now maintained by Dr A. McInnes at the University of Western Sydney (a.mcinnes@uws.edu.au).

3.2.2. Culture and Inoculant Production

Product finishing is essential for good quality inoculants and it involves the steps of culture multiplication, aseptic injection of broth culture into the peat, proper

maturation, and adequate packing. Either small or large fermentors made from glass or stainless steel can be used to grow cultures with growth conditions evaluated and optimized for each strain. Many media formulations have been described (Burton, 1967; Vincent, 1970; Roughley, 1970; Somasegaran and Hoben, 1994; Balatti and Freire, 1996), however, Stephens and Rask (2000) note that most of these were developed for general laboratory practice. They recommend a less nutrient-rich medium that is still able to support counts either at or exceeding 10^9 cells mL^{-1}. Several industrial by-products have also been used as carbon and/or nitrogen sources. They include corn steep liquor, proteolysed pea husks, malt extract, cheese derivatives, yeast extract, molasses, and casein hydrolysates. A common problem is of continuous supply and quality with these sources (Keyser *et al*., 1993; Walter and Paau, 1993; Stephens and Rask, 2000).

Table 3. Method of maintenance, main characteristics, and cell viability related to each method.

Method	Main characteristics	Viability
Agar medium	Medium usually with yeast, mannitol, and salts, kept at 5-6°C for periodic transfer; simple low-cost.	1 year
Agar medium	Covered with sterilized mineral or paraffin oil, kept at 5-6°C; simple and low-cost	2 years
Porcelain beads	Dry suspension of cells on sterilized porcelain beads, kept in a tube with dehydrated silica	2 years
Soil, peat or clays	Preferentially with high water capacity, ground, corrected for chemical properties, and sterilized	2-4 years
Paper strips	Paper strip or disk saturated with a bacterial suspension and dried, kept in the refrigerator.	6 months
Freezing	With temperatures ranging from -70ºC to -190ºC, in deep freeze or liquid nitrogen. Viability depends on the culture medium, freezing speed, freezing temperature, and type of cryoprotectant used; good viability has been shown in a number of collections after 15-20 years.	From months to several years
Lyophili-zation	Viability depends on the physiological state of the culture, cell concentration, medium, and lyophilization rate; can be kept at room temperature for years, but not much information is available.	From months to several years

An initial inoculum of 0.2-1% or more is used with most fermentors to ensure sufficient growth while reducing the possibility of contamination. Transfers between fermentors may be necessary to obtain the final volume of broth needed. More details about fermentation processes and types of fermentors can be obtained elsewhere (Walter and Paau, 1993; Balatti and Freire, 1996). The factors usually

controlled in culture production are the medium, temperature, agitation, pH, and aeration; inoculant batches should be checked for contamination at all steps. Under proper conditions, cell densities of 10^9 to 10^{10} mL^{-1} are usually obtained with dilution possible before injection into pre-sterilized peat carrier (Somasegaran, 1985; Keyser *et al*., 1993; Balatti and Freire, 1996). Before injection into the carrier, cultures must be tested for pH change, Gram-stain reaction, and the number of viable cells, and contamination must be assessed (Balatti and Freire, 1996). Injection into pre-sterilized peat must be done under aseptic conditions and, when thousands of doses are being manufactured, electronic injection is desirable. In peat inoculants, cultures are usually mixed to establish a 45-60% moisture content on a wet weight basis (Roughley, 1970). For non-sterile peat, either a rotating bowl or a concrete mixer is used to facilitate mixing during broth addition. For pure-culture peat, the inoculated bags are either manually or mechanically agitated to distribute the inoculum and to remove lumps.

The importance of a period of storage (for maturing and curing) after inoculation has been recognized for some time. Burton and Curley (1965) showed that rhizobia, which were allowed to grow and colonize the peat particles after inoculation, were able to survive on seeds in greater number and for longer periods of time than rhizobia freshly adsorbed in peat. Inoculants are usually held at warm room temperature to stimulate multiplication. Materon and Weaver (1985) noted ten-times more growth of *R. leguminosarum* bv. trifollii when peat carriers were stored for at least four weeks after inoculation before being utilized. After curing, the inoculant is usually maintained at 4ºC, however, the temperature used should be individually determined because the viability of some strains may decline at this temperature (Somasegaran, 1985). The storage conditions will also influence shelf life, so affecting cell viability, physiological characteristics (such as sensitivity to drying), and the time for colony and nodule appearance (Roughley, 1970; Burton, 1975; Revellin *et al.*, 2000; Catroux *et al*., 2001). Inoculant labeling should include product registration information and batch numbers, the strain(s) of rhizobia and other microbes included, if genetically modified microorganisms are included, the number of cells guaranteed by the manufacturer, and instructions for use.

3.2.3. Inoculant Quality Control

The quality control of inoculants prepared in pre-sterilized peat is easier because the enumeration of rhizobia can be done by simple plate count methods (Vincent, 1970). For non-sterile carriers, most contaminants will grow faster than the rhizobia and sometimes appear similar to them, which complicates the counting. For non-sterile carriers, selective media that contain antibiotics, heavy metals, bacteriocides and/or fungicides have been described (Vincent, 1970; Tong and Sadowsky, 1994; Gomez *et al*., 1995), but they are not equally effective for all strains. Plant infection, by MPN counts, should be carefully undertaken with rigid attention to detail. Results may vary not only with the number of serial dilutions, number of replicates, and the volume applied in each replicate, but also with the physiological state of the cells, the concentration of the inoculant, and the time allowed for plant growth (Lupwayi

et al., 2000; Catroux *et al*., 2001). MPN counts are both time- and space-consuming and require about 30 days for plant growth and adequate greenhouse or growth chamber space. For specific strains, methods based on serological properties may be used (Keyser *et al*., 1993; Lupwayi *et al.*, 2000), but other sophisticated methods to evaluate cell viability have been proposed (Catroux *et al*., 2001).

At a national level, quality control varies with country. It can be left to the discretion of the manufacturer, as in the United States and United Kingdom, or evaluated through an organization in which the manufacturers participate voluntarily, as in South Africa, New Zealand, and the Australian Inoculant Research and Control Service, or regulated through a governmental institution, as in Brazil (through the Ministry of Agriculture) and Uruguay. Both rhizobial concentration and the level of contaminants are important and the standards vary with the country. In Australia, Canada, Czechoslovakia, France, India, Kenya, New Zealand, Rwanda, South Africa, Russia, Thailand, The Netherlands, and Zimbabwe, inoculants must contain 10^7-10^9 cells g^{-1} at manufacturing or mL^{-1} for shelf life, depending on the country. In these countries, the inoculants must also either be void of contaminants or with no contaminants at the 10^{-6} dilution (Smith, 1992; Lupwayi *et al*., 2000; Stephens and Rask, 2000). In the countries of Mercosur, the manufacturers can be legally charged if the inoculants contain less than 10^8 cells g^{-1} or mL^{-1} of inoculant (for shelf life), with no contaminants permitted at 10^{-5} dilution.

It is well known that large inocula favor survival in greater numbers (Burton, 1976) and, as the retained inoculum may be as low as 5-10%, it is recommended that inoculant standards are based on numbers delivered per seed (FAO, 1991). Figure 3 shows the relationship between the number of cells applied per seed and the number of nodules produced in a field trial performed in Brazil.

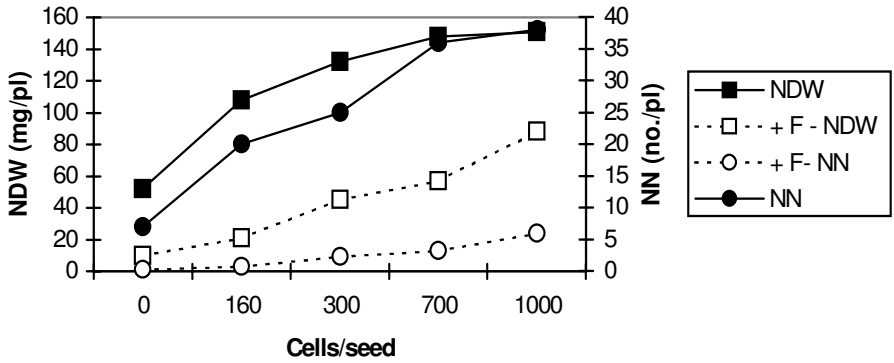

Figure 3. Nodule number (NN) and dry weight (NDW) of soybean cv. BR 16 inoculated with different concentrations of Bradyrhizobium elkanii SEMIA 587 with or without fungicide (F, thiram + thiabendazole).Experiment performed in an oxisol of Ponta Grossa, State of Paraná, Brazil, with less than 10 cells g^{-1}. After M. Hungria and R.J. Campo (unpublished).

Standards usually dictate a minimum of 10^3 rhizobia seed^{-1} for small-seeded legumes (such as clovers, *Trifolium* spp. and alfalfa, *Medicago sativa*), 10^4 rhizobia seed^{-1} for medium-sized seeds (such as pigeonpea, *Cajanus cajan*), and 10^5 rhizobia seed^{-1} for larger-seeded species (such as soybean, peas (*Vicia* spp.) and beans) (Thompson, 1980; Keyser *et al*., 1993; Smith, 1992; Lupwayi *et al*., 2000). In France, with a long tradition of inoculant legislation, a minimum of 10^6 cells seed^{-1} is required for larger-seeded species; a similar level has been proposed in Canada (Lupwayi *et al*., 2000). In Brazil, where the requirement has been slowly changing over time, the concentration recommended for large seeds is of 600,000 rhizobia seed^{-1}. The importance of cell number in the inoculant, when either conditions are adverse or the soil already contains ineffective rhizobia, has often been reported. For soybean, Weaver and Frederick (1974) indicated that cells of the added inoculant needed to outnumber resident rhizobia by 1,000-fold. Furthermore, in nine field sites in New Zealand, clover nodulation in pasture increased from 5% to 66% with an increase in the inoculation rate from 0.2×10^3 to 260×10^3 cells of *R. leguminosarum* bv. trifolii per seed (Patrick and Lowther, 1995).

4. INOCULANT APPLICATION

A number of different methods of inoculation are used by farmers, but not all are equally effective. In particular, the practice of mixing peat inoculant with the seeds in the planter box, although popular because of the ease with which it can be accomplished, is not recommended. Most of the inoculant will not stick to the seeds, resulting in non-uniform distribution; inoculant left on the box can gradually plug seeding tubes, so delaying sowing (Cattelan and Hungria, 1994). The sprinkle method, in which seeds are first sprinkled with water and then with dry inoculant powder, is little better. The dry seeds quickly absorb the water, not much is left as an adhesive for the peat, and most of the inoculant does not adhere to the seeds.

4.1. Slurry method

The slurry method is recommended for seed inoculation is the slurry method. First, the inoculant is mixed with a solution containing adhesive, then this slurry is applied to and mixed with the seeds until a uniform coverage is achieved. Seeds are then allowed to dry under cool conditions before sowing. Adhesives ("stickers") commonly used include sucrose (usually as a 10% solution), fungicide- and bactericide-free gum arabic (usually at 40%), carboxy-, methyl-ethylcellulose and methyl-hydroxypropyl cellulose (about 2-4%), or home-made gums prepared from cassava (*Manihot* spp.), starch, or wheat (*Triticum* spp.) flour (Roughley, 1970; Elegba and Rennie, 1984; Faria *et al.*, 1985; Cattelan and Hungria, 1994; Horikawa and Ohtsuka, 1996a). Either carpenter's glue or wallpaper adhesive have also been used, but some of these products contain fungicides that can kill the rhizobia. Slurry volume, in the case of soybean, should not exceed 300 ml per 50 kg of seeds. Peat inoculant is applied at rates of 1-10g kg^{-1} seed, depending on the cell concentration.

Table 4 shows the importance of sucrose solution, the most popular adhesive in Brazil. Without it, almost 50% of the peat inoculant was lost as the seeds dried.

Table 4. Slurry method of inoculation: Effect of different sucrose solution concentrations in the adherence of a peat inoculant onto the seeds and on grain yield of soybean cv. BR-37. The adhesive and the inoculant (10^8 cells g^{-1}) were applied at a dose of 300 mL of sucrose solution per 500 g of inoculant per 50 kg of seeds. Experiments carried out in Londrina and Ponta Grossa, State of Paraná, Brazil, in soils with established populations of soybean bradyrhizobia[1].

Sucrose concentration	Inoculant adherence (%)	(g)	Grain yield (kg ha^{-1}) Londrina	Ponta Grossa
0	48.2 b[2]	241.0	2,692 ab[2]	2,312 a[2]
10%	91.5 a	457.4	2,952 a	2,290 a
15%	92.0 a	460.0	2,568 b	2,460 a
20%	88.0 a	440.0	2,680 ab	2,393 a
25%	80.9 a	404.5	2,710 ab	2,363 a
CV (%)	13.0	13.0	10.7	13.7

[1]After Brandão Junior and Hungria (2000).
[2]Data for each site represent the means of two experiments, each with six replicates, and when followed by the same letter, within the same column, do not show statistical difference (Duncan, $p \leq 0.05$).

In this experiment, it is also important to emphasize that the use of sucrose did not result in either seed diseases or in changes of seed vigor (Brandão Junior and Hungria, 2000). Sucrose may also act as a cell protectant, increasing cell viability, and allowing the storage of the inoculated seed under dry, cool conditions for periods of up to a week before sowing (Burton, 1975; Peres *et al.*, 1986). One important factor not often considered is the quality of the water used to make the slurry. Many farmers use containers previously used for fertilizers or fungicides, both of which are toxic to rhizobia. A second problem, also evident in Table 2, is that, although the peat inoculant may be of high quality, the rate of application may be too low. As a result, the number of cells applied $seed^{-1}$ may be inadequate and some of the benefits of inoculation lost.

Because of the time consumed in slurry inoculation at sowing, low-cost machines, which have separate compartments for the peat inoculant and slurry, have been developed in Brazil. The seeds may also receive liquid fungicides and micronutrients (Hungria *et al.*, 2001b). These machines are designed for 60 bags of seeds (50 kg bag^{-1}) h^{-1}.

4.2. Seed Pelleting

Seed pelleting is used where conditions at sowing are either less than desirable (either high temperature or low soil pH) or the seed is sown from the air. In this procedure, the seed is slurry inoculated using a strong adhesive, such as 40% gum

arabic, it is then rolled in very finely ground calcium carbonate or rock phosphate or clay to form the pellet (Roughley, 1970; Burton, 1975; Smith, 1992; Thompson and Stout, 1992; Horikawa and Ohtsuka; 1996b). In Australia, specific micronutrients were also added to the seed-coating material and usually showed good results in acid soils; however, negative results were obtained with Mo and Co added to seed (see Table 6). The amount of seed-coating material and adhesive depends on the seed size. Another variation in this method is the use of mineral microgranules amended with nutrients and inoculated with either peat or liquid inoculants. Fouilleux *et al.* (1996) reported a significant increase in both early nodulation and N grain content, and better survival of *Bradyrhizobium* in soil undergoing desiccation, using this procedure.

4.3. Pre-inoculation

Although rhizobial numbers are usually greater on freshly inoculated seeds than on seeds that have been preinoculated for subsequent sale (Rice *et al.*, 2001), pre-inoculation of seed can be useful either when sowing large areas in a short time or when the weather is unstable. For forage legumes, such as alfalfa (*Medicago sativa*), cell viability can be maintained even when pre-inoculation is performed several months before planting (Smith, 1992; Rice *et al.*, 2001). However, only highly reputable seed companies should be considered for the purchase of pre-inoculated seed because rhizobial numbers seed^{-1} can be dramatically reduced in pre-inoculated seed that has been improperly stored (Thompson *et al.*, 1975). An alternative, which has been used to increase cell viability, is pre-inoculation for sowing within, at most, 10 days of seeding. Even here, the quality of the product can be erratic (Brockwell and Bottomley, 1995). Vacuum processing and the use of adhesives with alfalfa can also markedly decrease both the viability of cells and nodulation, even when stored at cool conditions (Horikawa and Ohtsuka, 1996a; 1996b).

4.4. Soil Inoculation

For leguminous crops such as soybean, dry beans, and particularly peanut, a disadvantage of seed inoculation (with either peat or liquid inoculants) is the incompatibility between inoculant strains and seed-applied fungicides, insecticides, or micronutrient preparations. Another constraint is the limited number of cells that can be applied to small-seeded legumes, particularly under difficult seeding conditions. It is well known that inoculants should be placed as close as possible to the seeds because the movement of rhizobia in soil is limited (McDermott and Graham, 1989). Chamblee and Warren (1990) reported a lateral movement of rhizobia of only 15 cm in an 11-month period.

To overcome the limitations described above, the inoculant can be applied directly to the seed furrow in the soil as granules, peat, or liquid, separated from the seeds at planting time. The inoculants are not mixed with fertilizer, which can be injurious to the rhizobia, but separately banded into the soil. A disadvantage of this

procedure is the higher cost because the quantity of inoculant used is higher than that used for seed inoculation. For peat inoculants, the rate of application is usually either 6-20 kg ha^{-1} or 1 g m^{-1} of row (FAO, 1984; CIAT, 1988) and usually the cost limits its applications (Walter and Paau, 1993). Gault *et al.* (1982) mentioned a variation of the standard seed-applied peat-powder method, which consists of suspending peat in water, screening the suspension to remove large peat fibers, diluting with water in a spray tank, and spraying the slurry into the furrow. The inoculation of soybean grown in a first-year area through irrigation with a peat slurry at rates of 2, 4, 6 and 8 kg ha^{-1} also resulted in good nodulation (Smith, 1992).

Granular inoculants may use peat preparations milled and sieved to provide particles between 0.35 mm and 1.18 mm in size. These absorb the culture rapidly and, after being cured, flow uniformly through a granular applicator. Such granular peat preparations deliver at least 10^{11} cells ha^{-1} and perform comparably to seed-applied inoculants (Smith, 1992; Lupwayi *et al.*, 2000).

Broth inoculants usually packaged in dispenser bottles can also be delivered directly into the seed bed, using an inoculant tank, a pump, a manifold, and capillary tubes to deliver the liquid culture. The equipment is not expensive and is in use in Australia, the United States, and Brazil (Hely *et al.*, 1976; Brockwell and Bottomley, 1995; Campo and Hungria, 2002b). The depth at which the liquid inoculant is placed is also important, *e.g.*, soybean nodulation was superior when the inoculant was applied either to the seed in the furrow or 2.5 cm below the seed as compared to application at both 5.0 cm and 7.5 cm below the seed (Smith, 1992). In Brazil, to avoid toxicity from seed-applied micronutrients and fungicides, Campo and Hungria (2002b) had to use eight-times more liquid inoculant in the seed bed, and up to ten-times more is recommended by Urbana Laboratories (2002), indicating that the procedure is useful only under specific conditions.

5. FACTORS AFFECTING THE SUCCESS OF INOCULATION

5.1. Effects of Inoculation with Selected Strains in the Presence of an Established Population

We will not discuss the soil and environmental factors that can affect the success of inoculation. Some of those factors have been reviewed (*e.g.*, Cattelan and Hungria, 1994; Hungria and Vargas, 2000) and will be discussed elsewhere in this volume.

There are numerous reports in which the use of inoculant-quality strains, which were applied to legumes in soils with a low level of soil N and few indigenous rhizobia, resulted in measurable benefits in terms of nodulation, N accumulation, plant biomass, and grain yield. Benefits are much less common, however, in soils having either indigenous or established rhizobial populations. Populations of soil rhizobia as low as 20-100 cells g^{-1} of soil can limit the response of both soybean and common bean to inoculation (Dunigan *et al.*, 1984; Singleton and Tavares, 1986; Thies *et al.*, 1991; Nazih and Weaver, 1994). When soils are devoid of rhizobia and inoculation is needed to ensure adequate nodulation and N supply, farmers tend to inoculate and follow recommended procedures (Smith, 1992; Hall and Clark, 1995).

However, when the soil contains established rhizobial populations and inoculation is used as insurance, attention to proper inoculation practice can be limited.

A lack of benefit from inoculation in soils containing established populations of root-nodule bacteria should not be taken for granted. There are numerous reports of positive responses to inoculation of both soybean and common bean in Brazil in soils containing high numbers of indigenous rhizobia (Vargas *et al.*, 1992; Peres *et al.*, 1993; Nishi *et al.*, 1996; Hungria *et al.*, 1998; Hungria and Vargas, 2000; Hungria *et al.*, 2000a; 2000b; 2001b; 2002). When 13 experiments performed with soybean in several Brazilian states were analyzed, re-inoculation increased nodule number and dry weight, and nodule occupancy by the inoculated strain in the majority of studies. In nine of the thirteen experiments, re-inoculation significantly increased yield and total N content of grains (Hungria *et al.*, 2000b; see Table 5). When the results of field trials performed in other seasons and sites were added to this data set, the national mean increase in grain yield was estimated at 4.5% (Hungria *et al.*, 2001b). It is interesting to note that, with both common bean (Hungria *et al.*, 2000a; Mostasso *et al.*, 2002) and soybean (Campo and Hungria, 2000a), a further increase in both nodulation and yield was obtained by the re-inoculation with the same selected strains in the second year of application. More studies in this area are warranted.

Table 5. Mean and maximum percentage increases in yield (kg ha^{-1}) and total N in grains (kg N ha^{-1}) due to the inoculation with the combination of strains Bradyrhizobium elkanii SEMIA 587 and B. japonicum *CPAC 7 (=SEMIA 5080), when compared to the non-inoculated control. The increases were obtained in thirteen experiments performed in two Brazilian Regions, in soils with established population of soybean bradyrhizobia*[1,2].

Region	Grain yield (% increase)		Total N in grains (% increase)	
	Mean	Maximum	Mean	Maximum
Central-West	7.8	23	8.1	25
South	3.8	20	4.3	24

[1]After Hungria *et al.* (2000b).

[2]Each experiment was performed with four to six replicates and, after the multivariate analysis, the data presented in this table were statistically significant (Duncan, $p \leq 0.05$).

Differences between North American and Brazilian reports on response to inoculation in soybean and bean are intriguing. Several conditions in the tropics, including high soil temperature, acid pH, limited soil moisture, and perhaps even micronutrient availability, could affect the physiological properties and activity of soil rhizobia and explain, at least partially, the more positive results to inoculation evident in these regions (Hungria and Vargas, 2000). Differences could also be related either to the higher competitiveness of the established population of soybean bradyrhizobia in North American soils (Ham *et al.*, 1971; Weber *et al.*, 1989) or perhaps to differences in their distribution in soil (McDermott and Graham, 1989). However, as pointed out before (Santos *et al.* 1999; Hungria *et al.*, 2001b; Ferreira

and Hungria, 2002), it is also possible that the selection program in Brazil has paid more attention to the search for more efficient, competitive, and adapted strains.

5.2. Seed Treatment with Fungicides and other Agrochemicals

Seed treatment with fungicides has been an increasing problem that affects inoculation success in beans and soybeans. Insecticides and herbicides applied at sowing can also inhibit nodulation, N_2 fixation and yield (De Polli *et al.*, 1986; Evans *et al.*, 1991; Cattelan and Hungria, 1994; Campo and Hungria, 2000a; 2000b). In Brazil, more than 90% of soybean seeds are treated with fungicides, and cell death rates of up to 70%, after only two hours of contact with fungicides, have been reported (Table 6). Reduction of nodulation under field conditions has also been reported (Table 7). These effects are most severe in first-year cropping areas, mainly in sandy soils, but also in areas with established populations. Selecting strains with higher tolerance to fungicides is not easy (Evans *et al.*, 1989) and only limited efforts made to use compounds less toxic to the rhizobia.

Table 6. Percentage of Bradyrhizobium japonicum cell death rate in inoculated soybean seeds two hours after the treatment with fungicides or micronutrients[1].

Fungicide	Death (%)	Micronutrient[3]	Death (%)
Control[2]	0	Control[2]	0
Benomyl + captan	62	Sodium molybdate	46
Benomyl + thiram	41	Ammonium molybdate	41
Carbendazin + captan	60	Molybdenum trioxide	37
Carbendazin + thiran	64	Molybdic acid	78
Thiabendazole + captan	28	Commercial product 1	97
Thiabendazole + thiran	24	Commercial product 2	28

[1]Adapted from Campo and Hungria (2000a).
[2] Peat inoculant applied as a slurry (10% sucrose).
[3]The chemical compounds were applied at the doses of 20 g of Mo and 5 g of Co ha^{-1} and the commercial products according to the manufacturer.

One possible reason for the success of *Rhizobium tropici* strains in both Brazil and north-central Minnesota (Estevez de Jensen *et al.*, 2002) could be the marked tolerance of Type IIB, but not IIA, strains to both streptomycin and captan (B. Tlusty and P.H. Graham, unpublished). Integrated root-disease management strategies, including inoculation with biological control agents (Estevez de Jensen *et al.*, 2002), are needed. In Canada, fungicide-treated seeds require 2-3 times the usual inoculation rate (Agriculture and Agri-Food Canada, 2002). Vincent (1958) pointed out that peat may shelter the rhizobia from toxic substances in the seed coat. Consistent with this observation, when liquid inoculants were tested in the presence of several fungicides in Brazil, the cell death rate was higher than in the presence of peat inoculant (Campo and Hungria, 2000a; 2000b).

Table 7. Effects of seed treatment with systemic and non-systemic fungicides on both nodulation (NN, nodule number per plant) and decrease in nodulation in relation to the non-treated seeds (%) in experiments performed in either first-year cropping areas (Terra Roxa and Vera Cruz, State of Paraná, <10 cells g^{-1}) or areas with an established population of soybean bradyrhizobia (Cristalina, State of Goiás, 10^5 cells g^{-1} soil)[1].

Treatment	New areas				Old area	
	Terra Roxa (sandy soil)		Vera Cruz (clay soils)		Cristalina (sandy soil)	
	NN	(%)	NN	(%)	NN	(%)
Non- inoculated	1	-	5	-	34	-
Inoculated (I, peat, $3x10^5$ cells $seed^{-1}$)	23	0	34	0	44	0
I + Benomyl + Captan	6	74	26	24	-	-
I + Benomyl + Thiram	5	78	27	21	-	-
I + Benomyl + Tolylfluanid	5	78	25	27	-	-
I + Carbendazin + Captan	11	52	33	3	-	-
I + Carbendazin + Thiram	5	78	28	18	38	14
I + Carbendazin + Tolylfluanid	4	83	26	24	-	-
I + Carboxin + Thiram	14	39	29	15	33	25
I + Difenoconazole + Thiram	13	43	30	12	-	-
I + Thiabendazole + Captan	3	87	25	27	-	-
I + Thiabendazole+ Thiram	7	70	23	32	-	-
I + Thiabendazole + Tolylfluanid	5	78	32	6	34	23
C.V (%)	53	-	30	-	-	-
LSD (5%)[1]	3.6	-	6.2	-	-	-

[1]After Hungria *et al*. (2001b).
[2]Means of six replicates and values followed by the same letter do not show statistical difference (Test "t", $p \leq 0.05$).

The use of micronutrients, mainly Mo and Co, at sowing is also increasing as a result of both the continuous cropping of soils and the depletion of nutrient reserves. The importance of these two micronutrients for nodulation and yield in Brazilian soils cropped for several years has been consistently demonstrated with yield increases of up to 82% (Campo and Hungria, 2000a; 2002a; Campo *et al.*, 2000; Hungria *et al*., 2001b).

However, micronutrient preparations may also cause a dramatic decline in inoculant-cell survival on seeds (see Table 6 above; Date and Hillier, 1968; Graham *et al.*, 1974). Most commercial products tested in Brazil had a pH below 3.5 and toxic concentrations of nutrients, but some of the manufacturers used balanced formulas with neutral pH that decreased cell death (see Product 1, Table 6). A strategy to avoid this problem has been to produce seeds enriched in Mo, obtained by spraying seed nurseries with micronutrients after flowering (Campo and Hungria, 2002a).

5.3. Inoculation under Unfavorable Conditions

Under unfavorable conditions, peat is the most suitable carrier. It does not necessarily have to be applied to the legume for which it is finally intended. One approach has been to inoculate the previous crop and success was obtained when wheat (*Triticum aestivum*), rice (*Oryza sativa*), or maize (*Zea mays*) were inoculated in anticipation of subsequent seeding with soybean, green gram (*Vigna radiata*), or groundnut (*Arachis hypogeae*) (Diatloff, 1969; Gaur *et al*., 1980; Peres *et al*., 1989). In most instances, this inoculation of a surrogate host appears to have limited practical value. It could be important, however, where soil conditions mitigate against rhizobial survival on the intended host, *e.g*., with fungicide-treated seed. With rhizobial cell numbers on alternate hosts, such as wheat, increased by 10-fold to 300-fold following inoculation, it is possible that initial strain establishment under harsh environmental conditions might be better on cereals than on the legume host, but this remains to be determined. One situation where benefits are likely is in the revegetation of disturbed landscapes in the northern USA and Europe. The practice in Minnesota is to seed and rake in the cover crop before the first frost, but to broadcast legume seed on the surface. Rhizobia on such seed have to persist through the harsh winter period, with germination of the host legume delayed as much as 8-9 months after seeding (P.H. Graham and B. Tlusty, unpublished).

Inoculation of seedlings in either a greenhouse or nursery can be very useful in forestry to guarantee a successful nodulation (Keyser *et al*., 1993) and a combination of seed and soil inoculation may also be recommended for unfavorable conditions (Brockwell *et al*., 1985; Cattelan and Hungria, 1994). Post-planting inoculation can be either intentional or remedial (to correct poor nodulation). Among the alternatives mentioned in the literature are inoculation through a center pivot irrigation (Smith, 1992), sub-surface granular applications, and surface spray application (Rogers *et al*., 1982). A three-year experiment with soybean showed that, in addition to seed inoculation, a cover inoculation of the soil with irrigation water (at either the time of sowing or at the three-node V3 stage) with peat inoculant in suspension increased nodulation by 1.4-times to 2.4-times (Ciafardini and Lombardo, 1991). Both positive and negative results are mentioned in the literature as a consequence of post-planting inoculation and it seems that the success of this procedure is related not only to the method of application, but also to the timing of the application and the soil conditions during inoculant delivery. Given the life cycle of most crop hosts, it is not to be expected that post-emergent inoculation would be effective more than 30 days after sowing.

A major factor affecting the success of inoculation is the application of N-fertilizers. In high profit crops, such as soybean, there is an increasing pressure to sell N-fertilizers to the farmers. There are some reports of benefits due to the application of starter N (van Kessel and Hartley, 2000) but, in Brazil, doses as low as 20-40 kg of N ha^{-1} have substantially decreased both nodulation and N_2 fixation with no yield benefit (Crispino *et al*., 2001; Hungria *et al*., 2001b). van Kessel and Hartley (2000) analyzed 600 experiments performed over a 25-year period and concluded that the contribution of N_2 fixation to crop growth and yield decreased

after 1985 for soybean and after 1987 for common bean. They suggested that a major factor was the increased use of N-fertilizers worldwide. Therefore, more effort should be put into the use of inoculants and not fertilizers in legume crops.

It is also important to remember that, although the success of inoculation is related to good soil and crop management, what is good management in one environment may not apply in another. Thus, although higher rates of N_2 fixation have been reported under no-tillage conditions in the tropics and subtropics (Campo and Hungria, 2000b; Ferreira *et al.*, 2000; Hungria and Vargas, 2000; van Kessel and Hartley, 2000), the cooler spring temperatures under no-till conditions in the northern USA and Canada can delay nodulation and perhaps inhibit *nod*-gene expression (Zhang and Smith, 1996). In places where production or utilization of inoculants is limited, *e.g.*, in Asia and Africa (Eaglesham, 1989), new approaches should be sought for the use of promiscuous soybean cultivars that are able to effectively nodulate with indigenous bradyrhizobia (Mpepereki *et al.*, 2000).

5.4. Inoculation and Co-inoculation with other Microorganisms

Azospirillum is another diazotrophic and plant growth-promoting bacteria tested in multiple inoculation trials (Okon and Labandera-González, 1994). In trials in Mexico, yield differences in maize inoculated with *A .brasilensis* ranged from 15% to 78% (Y.Okon, pers. comm.) and more than two million ha cropped with maize is now being inoculated. Important results have also been obtained with micro-propagated sugarcane (*Saccharum officinalis*) inoculated with *Gluconoacetobacter diazotrophicus* in Brazil. We will not detail experiments involving co-inoculation, but several papers point either to synergism between co-inoculated rhizobia and plant growth-promoting bacteria (Smith, 1992; Okon and Labandera-González, 1994; Burdman *et al.*, 1996) or to benefits from inoculation with species of *Bacillus* having biocontrol activity (Araújo and Hungria, 1999; Estevez de Jensen *et al.*, 2002) among several others (van Elsas and Heijnen, 1990; Walter and Paau, 1993). Rhizobial inoculation can also stimulate other microorganisms, *e.g.*, root colonization by mycorrhizal fungus (Xie *et al.*, 1995), seedling emergence, and grain and straw yields of lowland rice (*Oryza sativa* L.) (Biswas *et al.*, 2000). Undoubtedly, there is a need for a range of additional studies to integrate the use of these different organisms, either alone or in combination, in agriculture.

6. MAIN CONCLUSIONS

The advances made in recent years have shown that it is possible to obtain inoculants with high rhizobial counts, which are free of contaminants and with a longer shelf life. Alternative carriers and technologies of inoculation have also been identified. Used appropriately, inoculants prepared using these methodologies can be important to agricultural sustainability, particularly in those countries where leguminous plants play a key role in the economy. Brazil, for example, has benefited enormously from an emphasis on nodulation and nitrogen fixation in crop and pasture species. However, the potential benefits of inoculation are often limited

by the poor quality of inoculants either available in the market or used by farmers. Further improvements in the technical requirements for improved inoculant production and in their quality control are needed. The transference of existing or improved technologies to different agro-ecosystems can also be limited by political decisions and burocracy in extension agencies. Better communication and interaction between scientists, extension agents, and farmers is also needed as pointed out by Hall and Clark (1995) for Thailand and by Marufu *et al.* (1995) for Africa. To convince politicians and governmental institutions of the benefits of inoculation, greater emphasis should also be given to economic studies (like those performed by Panzieri *et al*., 2000) that, in the great majority of cases, will demonstrate the value of using inoculants compared to chemical fertilizers.

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