Review article
Using Rhizobia in the 21st Century

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Abstract

Many regions of the world face agricultural problems in delivering rhizobial bacteria effectively or in fostering the development and efficient functioning of N_2-fixing root nodules. Today, new knowledge and molecular tools offer opportunities for addressing these difficult global issues. Africa has an additional resource in the form of wild and seldom-cultivated legumes, which offer possible new sources of agricultural legumes. Worldwide investigations that employ rhizobia as tools may define how bacteria colonize roots, form nodules on non-legumes, and promote plant growth by mechanisms other than N_2-fixation. Together these divergent activities offer hope for enhancing the use of rhizobia and other beneficial bacteria in 21st-century agriculture.

Keywords: African legumes, competition, inoculants, nitrogen fixation, *Rhizobium*

1. Introduction

Rhizobia in the genera *Rhizobium, Sinorhizobium, Bradyrhizobium, Mesorhizobium, Azorhizobium* and *Allorhizobium* are Gram-negative bacteria that reduce elemental N_2 to ammonia in association with a wide variety of leguminous plant species. The biological nitrogen fixation (BNF) contributed by rhizobia to agricultural crops, 43 Tg N/yr (Galloway et al., 1995), is used

immediately within the host plant. Commercial chemical fixation for fertilizer production reduces 78 Tg N/yr and has several disadvantages. Not all N fertilizer is available to plants because significant losses to the environment occur in the form of leaching and volatilization. In addition, large quantities of energy in the form of fossil fuel are consumed in the chemical reduction of N_2. Increasing the amount and efficiency of BNF by rhizobia can alleviate many negative aspects of fertilizer nitrogen production and use.

Legumes forming symbioses with rhizobia are special plants in agricultural and natural systems because BNF allows them to grow in the absence of soil N. The benefits of BNF for livestock and people who consume various parts of the legume are well-known. For example, cattle grazing on grass during the dry season in Malawi can lose weight, while those grazing on a legume-based pasture can gain as much as those supplemented with cottonseed meal (Thomas and Addy, 1977). From another part of the world, as much as 75% of the protein N in California dairy products comes from N_2 reduced by symbiotic *Sinorhizobium meliloti* in alfalfa, which is fed to cows. Maximizing the benefits of BNF in highly managed ecosystems is relatively easy compared with the challenges faced in using BNF under less uniform, more severe environments. Differing priorities govern various societies, but BNF is a natural process that can make a positive contribution to agriculture in all countries at any stage of economic development. The purpose of this review is to indicate how our current understanding of rhizobia may be used more effectively in the 21st century.

Relationships between rhizobia and a host plant are highly evolved interactions (Young and Johnston, 1989; Doyle, 1994; Provorov, 1998) that include molecular communication between the free-living organisms (Phillips et al., 1994; Dénarié et al., 1996), an infection phase when bacteria form root or stem-borne nodules (Hirsch and LaRue, 1997), and a final symbiotic phase when bacteria occupying specialized plant cells in the nodules use carbon compounds from the plant as a source of energy for reducing N_2 (Brewin, 1998; Kahn et al., 1998). Our understanding of these processes has increased greatly in the past 20 years (Long, 1996; Schlaman et al., 1998), and such information will be important for maximizing the benefits of BNF in agriculture. In the same period, however, much less has been learned about many uncultivated legumes that offer hope, through their association with rhizobia, for improving agriculture in many regions of Africa. For example, almost 200 species of tropical legumes were identified in 1979 as being underutilized, potentially important resources for the future of Africa (National Academy of Sciences, 1979), but today many of these plants are still largely unknown. Indeed, in some cases their rhizobial partners, if any, have not been examined. Assessing and understanding these agricultural resources remain important tasks at the end of the 20th century.

2. Rhizobia and Legumes: Special Partners

Many bacteria are capable of reducing N_2, but only rhizobia, *Frankia* actinomycetes, and certain cyanobacteria form specialized associations in which fixed N is available directly to the plant host. Of these three groups of bacteria, rhizobia are the most important for traditional agricultural enterprises because they form BNF symbioses with many cultivated grain and forage legumes. Other BNF bacteria occur as free-living cells in the rhizosphere, but they use the reduced N for their own growth and release N only as individual cells die. These associative BNF organisms can stimulate plant growth, but such effects generally are attributed to other bacterial products that have beneficial effects on the plant (Okon and Labandera-Gonzalez, 1994). Recent evidence shows that sugarcane harbors bacteria that contribute to plant growth through N_2-fixation (Boddey, 1995), and an increasing interest in endophytic bacteria may yet identify other BNF microorganisms that release reduced N into plants (Triplett, 1996; Hallmann et al., 1997). These facts, as well as our increased understanding of rhizobia, have renewed interest in extending BNF to non-legumes, but currently the symbiosis between legumes and rhizobia remains the most important example of BNF in agricultural systems.

Despite the recognized benefits of rhizobia and legumes, the effective use of these symbiotic partners is constrained by many factors in agricultural situations. For example, high temperature, low pH, saline soils, and limited availability of water often stress agricultural plants, including legumes. Rhizobia, too, face numerous adverse conditions as they move from a commercial fermentor, into an inoculum, and finally reach the roots of legumes growing in soil. While some of these difficulties can be overcome using educational and financial resources, solutions often are restricted by the biological materials available. Thus one resolution of these problems involves either modifying current legumes and rhizobia or finding new organisms that already tolerate particular environmental stresses. Both approaches are important.

3. African Legumes for the Future?

One valuable source for new legume crops is the African continent. Africa has a rich flora in general, and portions of the continent have plant species that are not present elsewhere. The Cape Peninsula in South Africa, for example, has seven endemic legumes (Trinder-Smith et al., 1996). One novel South African legume, *Aspalathus linearis* subsp. *linearis*, fixes N_2 in association with *Bradyrhizobium* spp., and its leaves are used to make a caffeine-free medicinal

tea (Muofhe, 1997). Whether there is a wider market for products based on indigenous African legumes remains to be determined. Many, but not all, African legumes have been tested for their capacity to form root nodules, and the results of those studies have been published in a series of reports (Grobbelaar et al., 1983). Other workers have assessed the rhizobial requirements for African legumes relative to rhizobia isolated from various parts of the world. For example, numerous indigenous *Trifolium* species found in southern Africa often show a preference or specific requirement for local rhizobia (Strijdom, 1998).

Studies of African legumes can have at least two significant effects. On one level they can make novel observations relating to the basic biology of BNF, which stimulate scientists around the world. A good example of this phenomenon was the work on *Sesbania rostrata* by Dreyfus and Dommergues (1981), which was expanded into new and interesting discoveries on rhizobial formation of stem-borne nodules by workers in many different laboratories (Boivin et al., 1997). Second, wild or semi-cultivated legumes from various parts of Africa, which show good BNF, can be selected, bred, and managed appropriately as new crops in specific African environments. Eventually some of these legumes may play an important role in world agriculture, just as *Vigna unguiculata* has spread globally from its center of origin on the African continent.

African legumes fix significant amounts of N_2 in some environments but less in others (Table 1). More extensive surveys indicate rhizobia associated with grain and tree legumes in Africa fix total amounts of N_2 that are comparable to those measured for legumes in other regions of the world (Peoples and Craswell, 1992; Peoples et al., 1995; Dakora and Keya, 1997). At any one site the total BNF and, more generally, the total agronomic yield reflect the legume and rhizobia present, the prevailing environmental conditions including especially soil quality, and the farming practices. Optimizing the relationships among those factors in the next century will be an important challenge, assuming that any other problems that may exist relative to infrastructure, land tenure, and economic viability of the producers are solved.

It is difficult to know how many of the plant resources native to Africa have been exploited fully, but one can easily conclude that an unknown potential exists. A report in 1979 highlighted nearly 200 underexploited legumes that might contribute to African agriculture (National Academy of Sciences, 1979). A survey of the literature published since that date suggests that some species have attracted scientific interest, but little new information is available on others (Table 2). It is still not clear whether some of these species are nodulated by rhizobia, and that must be one of the first priorities for investigators (Dakora, 1995). The numbers of articles noted in Table 2 for some species must be interpreted with caution. For example, the 91 references

Table 1.   Estimates of BNF in various African legumes

| Plant | Site | Crop N | N$_2$ Fixed | | Reference |
| | | | Portion | Amount of total | |
		kg N/ha	%	kg N/ha	
Food crops					
Cowpea	Kenya	92–94	26–35	24–39	Ssali & Keya, 1984
Cowpea	Ghana	226	89	201	Dakora et al., 1987
Cowpea	Nigeria	82	61	50	Eaglesham et al., 1982
Peanut	Ghana	128	79	101	Dakora et al., 1987
Soybean	Nigeria	255	89	227	Eaglesham et., 1982
Common bean	Kenya	128–183	16–32	17–57	Ssali & Keya, 1986
Woody browse					
Sesbania rostrata	Senegal	214–230a	36–51	83–109a	Ndoye & Dreyfus, 1988
S. sesban	Senegal	54–100a	13–18	7–18a	Ndoye & Dreyfus, 1988
Leucaena leucocephala	Nigeria	288–344b	34–39	98–134b	Sanginga et al., 1989
Acacia holosericea	Senegal	10–20c	30	3–6c	Cornet et al., 1985

a in 60 days; b in 6 months; c in 6.5 months.

Table 2.   New information on African legumes previously identified as underutilized[1]

Legume	Region	Domestication	Recent reports[2]
Bauhinia petersiana	Zambia, Southern Africa	wild	5
Macrotyloma geocarpum	Africa-wide	cultivated	14
Sphenostylis stenocarpa	Eastern Africa	semi-cultivated	81
Tylosema esculentum	Botswana, Namibia, Kenya, South Africa	wild	15
T. fassoglense	Eastern/Southern Africa	wild	1
Vigna fischeri	Malawi, Zambia, Kenya	wild	0
V. lobatifolia	Angola, Namibia, Botswana	wild	1
V. reticulata	Malawi, Zaire	wild	4
V. subterranea	Africa-wide	cultivated	208
V. vexillata	Africa-wide	wild	91

[1]Mentioned in a report by the US National Academy of Sciences (1979). [2]Articles published from 1979 to 1998, as noted in AGRICOLA or CAB databases.

dealing with *V. vexillata* did not all represent attempts to develop the plant as a new crop. Rather, much of the interest in this species concerned the possible transfer of novel genes for insect resistance from *V. vexillata* to the agronomically important *V. unguiculata*. Obviously this type of interest is also an important and valid use of wild plant species.

Unexploited African legumes have many possible uses. Some plants in Table 2, like *S. stenocarpa*, *T. esculentum* and *T. fassoglense*, produce both edible tubers and seeds. Others are known primarily for either tubers or seeds, but not both. In nearly all cases, foliage can be used by animals, and, as cover crops, the plants can stabilize soils and control erosion. It probably would be premature to exclude any of these species from further study, but persons who are interested in this area must be aware of several facts. First, a new crop generally becomes widely grown because one person has dedicated many years of work to developing and popularizing it. Such narrow focus and dedication in one person is seldom nurtured by market forces or modern educational institutions; it must come from within an individual. Second, although molecular technologies available today may speed the development of new crops, the traditional process of selection, testing, breeding, and optimization of agronomic management still requires a competent organismic biologist. These facts eliminate many scientists as potential leaders in the development of new crops and suggest that a team of individuals with complementary skills is needed. Few such teams are currently working on new legume crops, but the situation could change if a severe shortage of food increases the financial resources available for solving agricultural problems.

4. Solving Rhizobial Problems

Increases in our knowledge of rhizobia offer opportunities for improving agricultural symbioses only if the bacteria can be used successfully to form root nodules on legumes under field conditions. Given that establishing effective *Rhizobium*-legume symbioses justifies to some extent the activities of many scientists around the world, it should be disturbing to read a recent analysis from several scientists known for their experience with rhizobial inoculants (Brockwell et al., 1995). Those workers concluded that "90% of all [rhizobial] inoculant has no practical impact whatsoever on the productivity of the legumes for which it is used"! This review does not debate that dismal judgement but, rather, suggests opportunities that are available for overcoming two causative problems: inoculant quality and competition with rhizobia already present in the soil.

Delivering the best bacteria

Rhizobia have been applied to legume seeds for most of the twentieth century. The problems associated with that process have been outlined by others (Brockwell and Bottomley, 1995) and will not be discussed in detail. Traditional methods of searching for effective rhizobia and testing them critically under both controlled and field conditions have produced collections of elite rhizobia suitable for most inoculants. It seems probable that similar procedures will work if new legume crops are introduced to Africa. In those cases where new agronomic opportunities are compromised by special edaphic stresses, effective rhizobia often have been collected from soils in another part of the world suffering from the same conditions. One recent example was the isolation of elite, acid-tolerant *S. meliloti* strains from Sardinia for nodulation of annual medics in Western Australia (Howieson and Ewing, 1986). An analogous strategy should help solve many future rhizobial problems encountered in Africa.

Nearly all improvements in the inoculant industry have been directed toward supplying larger numbers of rhizobia to a young legume seedling. This approach is based on classic data showing that very high numbers of rhizobial cells are required in inoculants before the applied strain competes effectively with indigenous rhizobia (Sadowsky and Graham, 1998). Suitable commercial inoculants prepared with diverse physical processes are supplied to seeds in liquids, gels or peat (Brockwell and Bottomley, 1995; Bashan, 1998). Appropriate methods are identified by their success in forming root nodules under standard field conditions. In some instances a positive control using adequate mineral nitrogen fertilizer is available for comparison, but in many cases this measure of maximum performance is technically difficult to include in a randomized agronomic experiment. For this reason, it is often easy to say an inoculant is effective or ineffective, but it is usually impossible to conclude that an inoculant helped the legume achieve its full agronomic potential. It is important to answer this latter question for legumes growing under any type of harsh environmental conditions. After successfully nodulating a seedling with rhizobia, the bacteria become a more integral part of that agronomic ecosystem. In the best cases, effective rhizobia will persist until a new crop of the same legume germinates (Date, 1991), and this result helps growers minimize the costs of future legume production. It also contributes substantially to the problem of introducing new, superior rhizobial strains.

New horizons in the inoculant industry are limited by the extremely low prices paid for the products. There is some interest now in mixed inoculants containing two species of microorganisms that interact synergistically (Bashan, 1998), and such products probably are both biologically and financially feasible. For example, rhizobia that promote growth by BNF could be

complemented with a second strain that controls a pest or enhances root hair development. Some believe, however, the most sustainable solution would be a genetic recombination of the two traits in a single strain, such as rhizobia that produce the *Bacillus thuringiensis* insecticide (Bezdicek et al., 1994). Low prices for the final product also reduce the attractiveness of including chemicals that serve as osmoprotectants (Miller and Wood, 1996), which might help increase rhizobial survival in peat inoculants. Other compounds that act in trace amounts to stimulate rhizobial growth, like biotin (Streit et al., 1996) or quercetin (Hartwig et al., 1991) or inducers of nodulation genes, which can increase alfalfa root nodulation by *S. meliloti* (Kapulnik et al., 1987; Hernández et al., 1995), may be affordable. All such compounds might be supplied more cost-effectively from a newly selected or created plant genotype that releases the desired molecule.

Establishing the desired association

Rhizobia supplied to legumes face major problems, first in simply surviving in an inoculant and then in competing with indigenous soil rhizobia to form root nodules. Relatively little is known about the genetic basis of rhizobial cell survival in inoculants. Rynne et al. (1994) hypothesized that survival in soil might be increased by a capacity to metabolize aromatic compounds derived from the humic fraction. If the concept were true, it could have important implications for survival of bacterial cells in a peat inoculum. Unfortunately, the concept was proved false when a Tn5 mutant of *Rhizobium trifolii* WU95 which was incapable of catabolizing particular aromatic compounds survived just as well as wild-type cells in the experimental tests (Rynne et al., 1994). Although the hypothesis was not supported by experimental results in that case, the power and validity of using a genetic mutant to test such a concept is a useful example for future rhizobial studies.

Recent analyses remind us that the greatest problem facing superior inoculants is competition from mediocre indigenous rhizobia (Streeter, 1994; Vance and Graham, 1995). This issue has been recognized for many years, but new experiments have reconfirmed that size of the indigenous rhizobial population is the major environmental variable determining which rhizobia occupy root nodules (Thies et al., 1992). The issues are complicated because both host plant genes (Triplett and Sadowsky, 1992) and bacterial traits (Maier and Triplett, 1996) clearly affect competition between inoculated rhizobia and indigenous soil strains.

One difficulty in identifying solutions to the problem of competitive indigenous rhizobia is that we have only a rudimentary understanding how rhizobia compete to form root nodules. Root colonization by rhizobia involves

many processes, including chemotaxis (Bauer and Caetano-Anollés, 1990), attachment (Vesper and Bauer, 1986; Smit et al., 1992; Vande Broek and Vanderleyden, 1995), antibiosis (Triplett, 1988) and growth. In many cases growth has been viewed as a consequence of chemotactic or attachment events that place bacteria in a better position to consume carbon substrates exuded by roots. It is probable, however, that mechanisms, which directly increase growth rate, will by themselves enhance competitiveness (Hartwig et al., 1991; Streit et al., 1996). Thus competitiveness really represents an integration of all events leading up to root nodule formation.

Many factors controlled by bacterial genes contribute to rhizobial competitiveness (Fig. 1). Production of the bacteriocin trifolitoxin is a well-characterized rhizobial trait that has excellent potential for increasing competitiveness of diverse rhizobial species (Triplett, 1990). Trifolitoxin functions as a natural antibiotic that slows growth of bacterial cells lacking resistance genes. The genes required for trifolitoxin production and resistance are now available on a plasmid (Kent et al., 1998), and careful tests with appropriate genetic controls showed trifolitoxin production increased the competitiveness of *Rhizobium etli* for rhizosphere colonization and root nodulation of common bean in non-sterile soil (Robleto et al., 1997). Stachydrine, a betaine found in relatively large amounts on seeds of alfalfa and many other *Medicago* species (Phillips et al., 1995), apparently contributes to *S. meliloti* colonization of alfalfa roots by serving as a carbon source because mutation of a gene required for stachydrine catabolism reduced competitive root colonization by *S. meliloti* (Phillips et al., 1998). Stachydrine is metabolized to proline, and mutations in proline dehydrogenase, an enzyme involved in proline degradation, also impair rhizosphere competitiveness of *S. meliloti* (Jiménez-Zurdo et al., 1997). Mutations in the synthesis of lipopolysaccharides or extracellular polysaccharides can delay root nodule formation, but such results could operate through effects on growth, rather than a specific effect on competition (Maier and Triplett, 1996). A more direct and interesting effect on competition was shown by a "hydrophobic" mutant (i.e. altered in an unknown surface trait, possibly related to polysaccharides) of *R. etli*, which colonized roots normally in single strain inoculations but was impaired greatly in the presence of the wild-type strain (Araujo et al., 1994).

It may be possible to create conditions that favor growth, root colonization, and/or nodule formation by certain rhizobia. One proposal is to use rhizopines to create a rhizosphere that favors particular rhizobia (Murphy et al., 1995). Rhizopines are compounds made by bacteroids of certain *S. meliloti* and *Rhizobium leguminosarum* bv. *viciae* strains. The same cells contain genes for catabolism of rhizopines, and it was proposed that such cells should be favored in the soil surrounding nodules containing the compounds. Thus one generation of rhizobial bacteroids might promote growth of their progeny cells in the
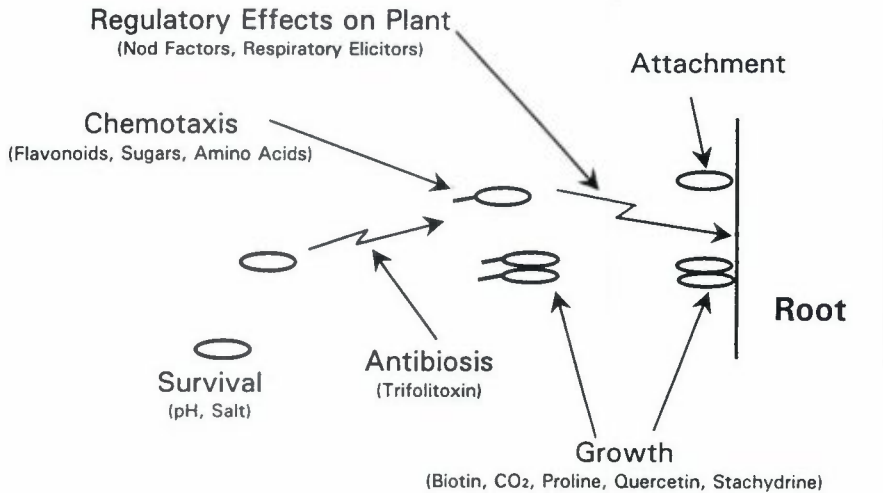
Figure 1. Processes affecting competitive root colonization by rhizobia. Genetic traits in the bacteria involving the specific factors named can influence root colonization and/or root nodule formation.

rhizosphere. A recent genetic test of the concept produced results that were not completely consistent with the hypothesis and more work is necessary to understand the actual benefits of these phenotypes in nature (Gordon et al., 1996). A related strategy in which the plant was transformed to produce opines favored competitive root colonization by *Pseudomonas* genotypes that had the capacity to catabolize those compounds (Savka and Farrand, 1997). To the extent that the goal of biasing the rhizosphere in favor of a particular bacterial strain can be achieved through genetic modification of only the bacteria, such traits may be incorporated into commercial rhizobial inoculants. If it is necessary to add chemical compounds to the inoculant in substrate amounts or to modify the host plant genetics, then the cost of production could exceed acceptable levels.

Often the establishment of an appropriate symbiosis is limited more by harsh environmental conditions than by competition from indigenous strains. In such situations it is possible to increase BNF greatly by producing or selecting rhizobial genotypes that can withstand the low pH, high salt concentration, or other unfavorable condition. For example, acidic soils create many problems for rhizobia, including those related directly to the effect of the excess protons on bacterial processes (Glenn and Dilworth, 1994) and those caused indirectly by the excess protons, such as toxicities of aluminum and manganese produced by their greater solubility at low pH or the decreased availability of calcium, molybdenum and phosphate at low pH (Flis et al., 1993). Solving all of the

problems associated with low pH will be difficult, but a greater understanding of actual proton tolerance is emerging (Tiwari et al., 1996a, b), and acid-tolerant strains with good BNF can be selected (Chen et al., 1991).

Normal development of rhizobial-legume symbioses is inhibited at many stages by salt stress, but overall, growth of legumes is far more sensitive to salt than growth of rhizobia (Zahran, 1991). Individual rhizobia may be sensitive to salt, but it generally is possible to find rhizobia that are resistant to salt levels that kill the plant host. Thus, if BNF in a particular rhizobial-legume symbiosis is to be extended into regions with excessively saline soils, then increasing the salt tolerance of the host plant must be an immediate goal. Alternatively, new legumes that tolerate the stress might be found. Biotechnology techniques available for improving crop legumes (Christou, 1994) should be helpful if new legumes are not available. Genes that increase tolerance to osmotic stresses could then be incorporated into the legume from any wild plant species. Many of those genes probably will contribute directly to increasing seed yield in grain legumes, separate from any benefits associated with maintaining N inputs from root nodules (Subbarao et al., 1995). In some cases, root nodules themselves are more sensitive to osmotic stresses than the same plant grown on combined N, and that situation may result from decreases in O_2 diffusion to the symbiotic bacteria in the root nodule (Walsh, 1995).

Improving rhizobial BNF

Modern molecular techniques now permit the construction of carefully designed rhizobial strains in which the actual BNF process should be more productive or metabolically efficient (Maier and Triplett, 1996). Favorite candidate genes for such improvements are the regulatory gene *nifA*, which enhances production of the nitrogenase enzyme complex, and *dct* genes to enhance transport of dicarboxylic acids into bacteroids (Scupham et al., 1996), or genes that may improve efficiency by recycling nitrogenase-generated H_2 (Kent et al., 1998). A versatile method for finding new beneficial genes or for increasing the copy number of known genes is random DNA amplification (Mavingui et al., 1997). Together these approaches may produce many rhizobial strains worthy of the field tests required to measure whether they improve BNF.

An early study showing that an undefined mutation enhanced N_2-fixation by *Bradyrhizobium japonicum* in pure culture and increased soybean seed yield significantly under field conditions (Williams and Phillips, 1983) has now been surpassed by the report that a genetically defined strain of *S. meliloti* increased alfalfa yield significantly (+3.8%) in field tests (Scupham et al., 1996). The elite *S. meliloti* strain RMBPC-2 contains modified forms of *nifA*

and *dctABD*, which optimize nitrogenase synthesis and uptake of C_4-dicarboxylic acids from the host plant. Although a 3.8% yield increase seems like a small improvement, it is typical of the increase in forage that is used to justify the release of a new alfalfa variety. These bacteria are now being sold in commercial inoculants. On the basis of these results, one can predict with confidence that other genetically improved strains of rhizobia will be constructed and used commercially in the future.

5. Extending the Rhizobial Example

As developmental interactions between rhizobia and their host legumes became more defined at the end of the 20th century, rhizobia have emerged as important tools for understanding how bacteria can be used more effectively in agriculture. In addition to their contributions to basic rhizosphere ecology through root colonization studies, these bacteria are being used to examine how root nodules can form on non-legumes and how plant-growth-promoting bacteria function. The single fact that best justifies these divergent uses is the availability of genetic data bases and techniques in rhizobia. One might suggest, for example, that studies of how *Frankia* bacteria naturally form nodules on non-legumes would be a more reasonable approach, but the relative lack of genetic data for *Frankia* severely limits the depth of such an investigation.

Several investigators have begun the arduous task of identifying molecular factors that prevent rhizobia from forming N_2-fixing symbioses with non-legumes (Ridge et al., 1992). The ultimate goal of that work is to use the BNF capacity of rhizobia in agriculturally important crops such as rice and other cereals. The feasibility of this objective is supported by the fact that *Bradyrhizobium* infects the non-legume *Parasponia* and forms lateral-root-like organs in which the cortex is filled with N_2-fixing rhizobia. This developmental process resembles legume root nodule formation in the sense that root cortical cell division occurs (Bender et al., 1987b) and certain rhizobial nodulation genes are required (Bender et al., 1987a). However, the bacteria do not infect root hairs. Instead a more general erosion of cell walls occurs and infection threads form as the underlying cortical cells divide. Parallels to this process can be found in work where treating *Brassica napus* roots with hydrolytic enzymes facilitated rhizobial infections (Al-Mallah et al., 1990). The absence of any significant N_2-fixation in the *Brassica* tissues suggests that additional interactions between the bacteria and plant host are required to achieve the symbiotic association found in *Parasponia*. One such limitation is the absence of the poorly understood barrier to O_2 diffusion that exists in legume root nodules. The effectiveness of that barrier is such that legumes

have evolved a second mechanism, the protein leghemoglobin, which facilitates diffusion of O_2 to rhizobia in the nodule. The positive evidence for rhizobial infection of *Brassica* and *Parasponia* has stimulated studies of how rhizobia might colonize and infect other plant species. These activities also have been energized by reports that certain rhizobial strains occasionally induce "nodule-like" structures on rice (reviewed in Ridge et al., 1992). One intriguing result from these studies is the observation that certain flavonoids stimulate a nodulation-gene independent intercellular colonization of roots in *Arabidopsis* (Gough et al., 1997), as well as in rice and wheat (Webster et al., 1997). A second is the observation, made possible only after significant preliminary work was completed, that rice roots perceive the presence of rhizobial nodulation signals in the rhizosphere (Reddy et al., 1998).

One can conclude from these results with non-legumes that many different levels of interactions between rhizobia and plants will complicate attempts to form functional rhizobial root nodules on rice. A corollary to that conclusion is that, although nature has conducted the experiment of trying to nodulate non-legumes for many years, not all possible combinations of bacteria and plants have been tested. Thus scientists interested in this area obviously have two types of work to do. On the one hand, they can define how rhizobia interact with various non-legumes in laboratory experiments. Alternatively, they can look for naturally occurring rhizobial symbioses in N-deficient soils where a wide diversity of non-legumes grow. *Parasponia* is a tropical genus, and it is not unreasonable to pursue this second approach in a region such as Africa.

A more general approach to extending BNF to non-legumes postulates that some N_2-fixing bacteria, which occur as endophytes in plants transfer significant amounts of reduced N to the host plant (Triplett, 1996). This type of study envisions such endophytes as being more amenable than rhizobia to fixing N_2 in other non-legumes as a result of some bacterial mechanism that protects the nitrogenase enzyme complex from O_2. *Azotobacter*, for example, carry their own mechanisms for protecting nitrogenase from O_2. Presumably endophytic BNF bacteria in plants have analogous mechanisms and could use them in other plants. The most promising result in this area is the demonstration that very significant amounts of BNF occur in sugarcane, a non-legume monocot (Boddey et al., 1997), but endophytic BNF bacteria have also been isolated from maize (Palus et al., 1996). In all investigations of this type the most important question to answer is whether or not the bacteria actually contribute reduced N to the plant.

One final example of how new knowledge from rhizobia may benefit agriculture in the 21st century comes from experiments suggesting that rhizobia can stimulate plant growth through mechanisms not involving BNF. In this case, two strains of *R. leguminosarum* bv. *trifolii* enhanced rice grain yield under a high level of N fertilization in Egyptian fields (Yanni et al., 1997).

Presumably BNF was inhibited by the fertilizer N, and the authors suggested rhizobia might stimulate rice yield by synthesis of either plant hormones or nodulation-gene-dependent products.

An alternative framework for explaining the beneficial effects of rhizobia on rice postulates that bacterial products, which are not plant hormones, induce plant responses that subsequently may involve hormones. The model for this thinking is based on the diverse responses shown by plant roots to low concentrations of Nod factors (Dénarié et al., 1996). For example, the known effect of *Azospirillum* on root hair development (Hadas and Okon, 1987) could result from a non-hormonal inducer molecule that affects differentiation of root epidermal cells. Another example is the currently unidentified *S. meliloti* molecule which, when applied to roots at possibly picomolar concentrations, increases alfalfa root respiration over an 8- to 12-h time period (Volpin and Phillips, 1998). The potential significance of this finding is evident in the fact that the same molecule, currently termed "compound D", triggered an increase in net photosynthesis which supplies carbon substrates for the additional respiration. As a result, measurable increases in plant dry weight accrue in a magnitude similar to the plant-growth-promoting effects of many bacteria (D. A. Phillips, unpublished data).

The novel finding that rhizobia can stimulate primary carbon metabolism in a plant host, even before N_2-fixation occurs, raise several questions: How could molecules like compound D evolve? How might such molecules contribute to agriculture in the 21st century? The initial driving force in the evolution of such a response probably was related to the fact that rhizobia require an exogenous supply of CO_2 for growth in addition to reduced carbon substrates (Lowe and Evans, 1962). Obtaining their necessary CO_2 from the plant helped rhizobia colonize roots more effectively than other soil bacteria and thus facilitated the subsequent evolution of root nodules. The possible agricultural benefits of compound D can be assessed only after the mechanism by which this molecule functions is defined. For example, whether the molecule is transported into the root and/or to the shoot is still unknown. However, the mere availability of a compound that affects key processes related to primary production, including root respiration and net photosynthesis (without yet separating effects on photosynthesis from those on photorespiration) offers a chance to test many fundamental and applied hypotheses. Preliminary results show that other bacteria produce molecules structurally related to compound D, and thus the full scope and importance of these new findings remain to be determined. Hopefully, these research activities will explain one mechanism by which diverse rhizosphere bacteria promote plant growth and, possibly, lead to the identification of novel compounds that have beneficial uses when they are applied directly to plants.

One benefit of rhizobial model systems may be the eventual consolidation of many desirable genetic traits into a few rhizobial strains. Thus it is reasonable to predict that, at some point in the 21st century, new strains of rhizobia will be engineered to contain not only the genes required to optimize root colonization and BNF but also other, perhaps non-rhizobial, genes that permit nodulation of non-legumes or that promote plant growth by mechanisms separate from BNF. Perhaps individual rhizobial strains that can nodulate all legumes will be available. Such bacteria would represent logical extensions of the biological knowledge and materials we have today, and they could be important contributors to 21st century agriculture.

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