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The role and future potential of nitrogen fixing bacteria to boost productivity in organic and low-input sustainable farming systems

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ABSTRACT

Biological nitrogen fixation (BNF) results from the interaction between a plant and diazotrophic bacteria. The bacteria are either free living in the soil or live in symbiosis with the plant. Despite biological nitrogen fixation offering a sustainable solution to nitrogen limitation in agricultural soils its use is in decline. Problems with this technology can arise for two major reasons. Firstly, the inappropriate use of diazotrophs with the expectation of achieving N_2 fixation. Free-living diazotrophs have been used as inoculants of non-legume crops for many years, however, their mechanism of action remains to be thoroughly characterised. While some may interact with crops to increase available N in soil, many achieve increases in crop yield through the production of plant hormones. This adds nothing to the soil N budget and increases in yields observed are often variable. The second problem occurs when legumes are used to increase soil N in combination with rhizobial symbionts. Frequently poor nodulation of the legumes is observed in the field even when inoculated with 'elite' strains of rhizobia. These observations are a consequence of one or more factors, including the use of low quality inoculants, the inability of the rhizobial inoculant to tolerate soil conditions, or their lack of competitiveness for nodule occupancy with indigenous soil rhizobia. These issues can be overcome by the use of more rigorous criteria in inoculant selection and production. The use of inoculants developed from indigenous soil rhizobia offers a tailor made solution to obtaining inoculant strains that are competitive in a particular soil with a specific crop. Here, examples of where this approach has been successful and the potential of this technology to increase the use of BNF in more marginal soils are discussed.

INTRODUCTION

Nitrogen (N) is the major limiting nutrient for crop growth in most agricultural soils. As a result, supplementing crops with inorganic N fertilisers has given an unprecedented boost to agricultural productivity such that today, the land area under wheat cultivation has shrunk by 4% but production has increased by 32% compared to 25 years ago (Smil 2001). Unfortunately these huge advances in productivity have not been without consequences, the prolonged applications of large quantities of fertiliser to soil are manifesting themselves in the degradation of the environment. In particular, the leaching of nitrates into ground and surface waters, and the emission of greenhouse gases from agricultural soils (Ridley et al. 2004).

In recent years the use of inorganic fertiliser has risen at the expense of biological nitrogen fixation (BNF), mostly because of increased usage in the developing world. Currently 2.4 billion people depend on synthetic fertiliser for protein (Smil 2001), but predictions of population growth in developing countries indicate that they will need an additional 15 million tonnes of protein nitrogen over the next 50 years. As a result the increasing use of intensive agricultural systems relying on inorganic N looks set to continue because its application gives a reliable boost to crop yield in a cost effective manner. It also increases productivity because land can be used to grow cereals continuously rather than requiring regular rotation with legumes to replace N as in the BNF model (Crews and Peoples 2004; Jenkinson 2001).

Unfortunately the consequence of intensive agriculture on the environment can be significant. It has been shown that in infertile tropical soils, organic matter decreases, rapidly resulting in erosion and desertification (Graham and Vance 2000). In addition, the cost of inorganic fertiliser is prohibitive to many farmers in the developing world. For example, over 90% of Nigerian farmers use inorganic fertiliser, but in the majority of cases they apply only half the amount that is recommended because of the costs involved (Sanginga 2003).

Despite the increasing use of inorganic N fertiliser the use of biological nitrogen fixation (BNF) is still widespread, indeed many intensive agricultural systems continue to depend heavily on BNF, notably in Australia and Brazil, where symbiotic N_2 fixation using forage and grain legumes has been successfully employed to increase soil N for a number of years. In developing countries, advances in plant breeding and inoculant technology are also offering farmers sustainable alternatives to costly inorganic fertiliser. Moreover, organic inputs are being promoted as a mechanism to enhance soil N in tandem with fertiliser application to increase the area of marginal soils available for cultivation (Sanginga 2003).

In this review, the current and future potential of N_2 fixing bacterial inoculants to increase crop yield are highlighted. The range of bacteria used as inoculants are discussed focussing on the intimacy of their relationship with the plant and their mechanism of action. I also address the problems constraining the effectiveness of rhizobia as an alternative to N fertiliser with emphasis on the viability of such inoculants and their persistence in the soil. Three factors are identified that limit their effectiveness: (i) poor quality inoculant with low viability; (ii) the inability of inoculant to compete with native rhizobia; (iii) inoculant which cannot tolerate the physical and chemical conditions in the soil. Finally I discuss current and potential approaches that may circumvent these problems. In particular, I advocate the use of rhizobial inoculants that have been selected to provide specific crops or cultivars in a particular soil type with a high quality, persistent and competitive inoculum.

SUCCESSFUL APPLICATIONS OF BNF IN AGRICULTURE

There are examples where BNF remains a significant contributor to N inputs in agricultural soils. For example, Australian agriculture embraced the use of BNF to address the low fertility of many of their temperate soils (Howieson and Ballard 2004), and legume rotations continue to be widely used accounting for the annual fixation of almost 5 million tonnes of nitrogen (Crews and Peoples 2004). Equally important is that all the legumes used in these agricultural systems, and their associated rhizobial symbionts are exotic to Australia (Baldock and Ballard 2004). This represents a significant achievement in the managed introduction of exotic plant and bacterial species into a novel milieau.

In the developing world BNF remains as an essential tool in the maintenance of soil fertility. Many subsistence farmers rely on it as the only mechanism to address N deficiency. As a consequence, work in sub-Saharan Africa has focussed on the development of crop varieties that are resistant to environmental stresses such as drought (Shisanya 2002) or can promiscuously nodulate with indigenous soil rhizobia (Musiyiwa et al. 2005). The latter approach is being applied to soya bean, resulting in an opportunity for farmers to access a cash crop without the requirement to purchase expensive inorganic fertiliser or commercial rhizobial inoculants (Mpepereki et al. 2000). The cultivation of soya bean in Brazil is also a success story for the introduction of an exotic crop into an agricultural system. Since the introduction of commercial cultivation in the 1960s the soya bean has become Brazil's premier agricultural export and the country is now second only to the USA as a producer of this crop. Moreover, Brazilian soya bean varieties rely predominantly of BNF unlike those used by the other major producers (Alves et al. 2003).

The application of BNF to agricultural systems has a number of advantages over the use of inorganic N inputs. For example it is a more sustainable technology, inorganic N fertiliser manufacture consumes 1.3 tonnes of oil equivalents per tonne manufactured (Howieson et al. 2000). BNF also provides a break in the crop rotation, reducing losses due to pathogen and pest damage and reducing the requirement for pesticide application. Although it is unlikely that in the foreseeable future the reliance of inorganic fertiliser will be supplanted by BNF an increased uptake of this technology, in partnership with other advances, such as cropping systems, plant breeding and soil management practices may ultimately allow a significant reduction on the reliance on inorganic fertiliser application.

PROBLEMS WITH BIOLOGICAL NITROGEN FIXATION

Although BNF has been widely used in many agricultural systems to boost crop yield, there is a reluctance among many farmers to adopt this technology at the expense of inorganic N application. This reflects wider concerns about the reliability and robustness of BNF. A key issue to be addressed is whether the use of BNF is effective in increasing the N available in agricultural soils. Typically legumes are grown in rotation with cereal crops, and after harvest the decomposition of the whole plant or the root material liberates biologically available N fixed by the legume into the soil. An increase in soil N will be observed only when fixed N input is greater than N removal in biomass or grain (van Kessel and Hartley 2000). In many studies, the effect of BNF are illustrated by demonstrating increases in crop yield, unfortunately this data can be confounded for several reasons. Many bacteria that are used as inoculants produce plant hormones that give significant increases in crop yield in the short term but have no effect on soil nitrogen (Andrews et al. 2003). Therefore, the use of isotopic techniques exploiting ^{15}N natural abundance or isotope dilution with labelled fertiliser addition to soil are considered the most reliable measure in the field. These studies have demonstrated an astonishing range of N_2 fixation in the field, for example, between 0-450 kg N ha^{-1} for soya bean (Unkovich and Pate 2000). Such variation reflects both differences due to temporal and spatial growing conditions but also problems with the methodology, that for example, fails in many studies to account for N fixed in legume roots that are constantly exuding and turning over N (Unkovich and Pate 2000).

As a result, the reliance on BNF is perceived by farmers as a much less reliable strategy than using inorganic fertiliser, primarily because of the variability in the amount of nitrogen fixed in legume based systems. This was illustrated in a recent study, that showed BNF in Australian forage legumes ranged between 9 to 36 kg fixed N t^{-1} shoot dry matter (Peoples and Baldock 2001). Also, the benefits derived from legume rotations reflect the different N economies of the legume employed. For example, a comparison of the potential benefit to subsequent cereal crops in SW Australia, indicated that lupin (*Lupinus angustifolius* L.) produced 68 kg N ha^{-1}, compared to 3 kg N ha^{-1} derived from field pea (*Pisum sativum* L.) (Unkovich et al. 1995). Finally, the effective application of BNF requires that farms maintain 20-50% of their land under legumes at any given time. However, the pressure for increased productivity from agricultural land, particularly in the developing world make such agricultural practices much less attractive (McKenzie and Hill 2004).

Another problem that is rarely articulated is that despite the environmental benefits that are frequently attributed to BNF when compared to the use of inorganic fertiliser, legume based systems are capable of causing environmental degradation. There is particular concern in Southern Australia, where the use of pasture legumes as a sustainable form of soil N management has been criticised. It has been argued that in combination with climatic conditions, BNF poses a significant risk for nitrate leakage into ground and surface waters (Ridley et al. 2004). Amelioration of this problem will require careful consideration of how N production can be optimally synchronised with crop uptake (McKenzie and Hill 2004). Soil acidification has also been observed after prolonged legume cultivation, as both legumes and rhizobia are sensitive to changes in soil pH this can have a significant impact on productivity (Slattery et al. 2001; Table 1).

BIOLOGICAL NITROGEN FIXATION IN AGRICULTURAL SYSTEMS

Understanding the relationship between the inoculant and the plant

The most commonly used bacteria to fix nitrogen in an agricultural context are those that form symbiotic interactions with legumes. These bacteria belong to a variety of genera but are collectively referred to as rhizobia (Howieson and Ballard 2004). Symbioses between rhizobia and legumes are the result of a complex series of signals exchanged between the plant and potential rhizobial symbiont in the soil. The plant secretes flavonoids that induce the expression of nodulation genes. The Nod-factors produced cause curling of root hairs which provide a route of entry into the plant via an infection thread (Broughton et al. 2003). The importance of these bacteria in legume rotations, particularly in Australia and the Americas, has resulted in a substantial body of literature devoted to the identification and characterisation of appropriate isolates to use as inoculants for forage and seed legumes that can effectively enhance crop yield and improve the N status of the soil. A number of bacterial strains have been developed as 'elite' inocula for particular legumes. In Brazil, for example,

Table 1. The impact of soil stresses on the effectiveness of commercial and stress tolerant inoculants in a range of legumes of agricultural significance.

Bacterium	Plant	Stress	Reference
Rhizobium PMA63/1 *Rhizobium* PMA403/1	*Acacia ampliceps* *Acacia stenophylla*	**Salt**; Inoculation gave significant improvements in seedling survival over controls in saline soil.	Shirazi et al. 2001
Bradyrhizobium RCR 3407 *Rhizobium* USDA 208	*Glycine max* L.	**Salt**; Salt-tolerant USDA 208 fixed more N_2 in saline soils than the salt sensitive RCR 3407. However, there was no significant difference between them in shoot or root dry wt. and nodule number.	Elsheikh and Wood 1995
R. leguminosarum bv. *viciae* strains	*Vicia faba* cv Fiord	**pH**; The persistence of *R. leguminosarum* bv. *viciae* in 3 Australian acid soils (pH 4.8-5.7) was poor. After 2 years, of the 8 introduced strains only 3 maintained populations in excess of $100g^{-1}$.	Carter et al. 1995
R. leguminosarum bv. *trifolii*	*Trifolium alexandrium* cv Elite II	**pH**; Variability in successful colonisation by commercial inoculants in alkaline Australian soils was related to poor survival post sowing, poor alkaline tolerance, poor plant rhizobial interactions and competition from indigenous rhizobia.	Denton et al. 2003
Bradyrhizobium WU425 (lupin)	*Lupinus angustifolius* cv Yandee *L. pilosus* P23030	**pH**; The 2 lupin species grow poorly on neutral and alkaline soils. As pH increased from 5 to 7.5 the number of nodules decreased by 50% or more. *Bradyrhizobial* isolates also decreased in the soil by an order of magnitude over the same pH range.	Tang and Robson 1993

Rhizobium tropici strains CIAT899 and PRF81 are recommended as inoculants for the common bean (*Phaseolus vulgaris* L.) (Mostasso et al. 2002). Similarly in Australian agriculture, that in recent years has seen the introduction of several new legumes, specific inoculant strains are sold commercially to promote N_2 fixation particularly when these crops are first cultivated in soils. This practice is necessary because the indigenous rhizobia are usually unable to form effective symbioses with exotic legumes (Howieson et al. 2000). In other agricultural systems, notably in Africa, an alternative strategy of breeding crops that can nodulate promiscuously with indigenous soil rhizobia has been adopted (Mpepereki et al. 2000). This latter approach reduces the economic burden on the farmer while still allowing the opportunity to promote soil fertility through N fixation.

There are also large populations of N_2 fixing bacteria such as *Azospirillum*, *Pseudomonas* and *Azotobacter* species free living in the soil and rhizosphere that do not form symbiotic associations with plants. Such bacteria have been used extensively as inoculants of dryland graminaceous crops since the middle of the 20^{th} century in an attempt to exploit their capacity to fix atmospheric N. However, evidence from large scale trials in Russia with *Azotobacter chroococcum* on wheat and barley (*Hordeum vulgare* L.) and studies across the world with *Azospirillum brasilense* and *A. lipoferum* on a range of graminaceous crops have failed to demonstrate a reliable positive effect on crop yield (Andrews et al. 2003). These data suggest that rhizosphere bacteria such as *Azotobacter* and *Azospirillum* act, not by providing the crops with additional N, but rather, via changes in root morphology and physiology (probably hormone induced). As a consequence there is increased mineral nutrient and water uptake from the soil and these effects result in greater crop growth and subsequently greater yield (Andrews et al. 2003).

In some cases, diazotrophic bacteria are found in intercellular spaces within inner root tissues of non-leguminous plants, and these bacteria have been described as endophytes. There have been claims that endophytic diazotrophs such as *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans* that colonise some tropical grasses, especially sugar cane (*Saccharum* spp.), wetland rice (*Oryza sativa*) and kallar grass (*Leptochloa fusca*) can provide, at least in part, some of N-needs of the plants from BNF. The identification of the bacteria engaged in this N_2 fixation remains an active area of research and many remain to be characterised, however, *Azoarcus* spp. seem to play a major role in N_2 fixation in kallar grass (Hurek et al. 2002), while *G. diazotrophicus* and *Herbaspirillum* spp. could be important in N_2 fixation in sugar cane. Caution needs to be applied to the interpretation of such data, however, as there remains some controversy over the nature of the interaction between the bacteria and plant. For example, *G. diazotrophicus* was among the first bacterium identified as 'symbiotic' with sugar cane (Dong et al. 1994). However, James et al. (2001) pointed out that no growth response, *in planta* nitrogenase activity or BNF was conclusively demonstrated. Moreover, whether the bacteria are actually present in significant numbers within the plant remains to be convincingly demonstrated.

At present the potential of endophytic bacteria remains to be established. Potentially they offer the opportunity to enable BNF to be extended into a variety of non-leguminous crops. Recently a study demonstrated that nitrogen fixation in wheat (*Triticum aestivum* L.) by *Klebsiella pneumoniae* could be unequivocally demonstrated (Iniguez et al. 2004). The response was observed only in a single cultivar (Trenton), however, the implications of this work are enormous if the mechanism by which the bacterium provided this benefit can be identified then the phenotype could be extended into other wheat cultivars. At present, however, it is the application of rhizobial–legume symbioses that represent the most mature and robust technology. The extensive literature on this topic provides not only a historical context, but illustrates the potential application of bacterial inoculant technology in sustainable agriculture. As a result I shall focus on these systems in the remainder of this review.

THE USE OF N FIXING INOCULANTS IN SUSTAINABLE AGRICULTURE

The application of rhizobial inoculants, capable of forming a symbiotic interaction with leguminous crops, is a cheap and in many cases effective mechanism to enhance nodulation and N_2 fixation. However, there are many reports that indicate inoculation can fail to achieve these responses. This lack of success can occur for a number of reasons including low viability of the inoculant, its poor persistence in the soil environment, or an inability to compete with the resident bacterial population. Despite the problems associated with the application of inoculants to leguminous crops, there are particular circumstances when inoculation does have clear benefits. These are when:

- There is a low indigenous population of N-fixing bacteria and limited N within a soil. An example of such conditions are the soils of the Cerrados of Brazil, where, as described earlier soil conditions are extreme, with low indigenous populations of rhizobia. As a result the nodulation of the common bean (*Phaseolus vulgaris* L.) is poor but can be improved by inoculation with *R. tropici* PRF 81 (Hungria and Vargas 2000).
- There is no history of legume cultivation in a soil or a legume is being cultivated that is exotic. This is clearly illustrated by the widespread cultivation of Mediterranean legumes in Australia. As indigenous rhizobia could not effectively nodulate these legumes exotic rhizobia were and continue to be imported to use as inoculants (Baldock and Ballard 2004; Howieson et al. 2000). Similarly in

sub-Saharan Africa, inoculation of soybean (*Glycine max* (L.) Merrill) with *Bradyrhizobium japonicum* increased yield from 500 to 1500 kg ha^{-1} (Mpepereki et al. 2000).

FACTORS CONFOUNDING THE USE OF N FIXING INOCULANTS

Poor quality inoculant with low viability

A decade ago it was estimated that 90% of inoculant has no practical effect on legume productivity (Brockwell et al. 1995). These observations reflected problems in the manufacture and utilisation of such products. Inoculants are typically manufactured as a bacterial suspension in a carrier, usually peat, however, in developing countries with no access to peat other carriers such as sugar cane pith can be substituted (Marufu et al. 1995). In many poor quality inoculants the carrier is not sterilised prior to addition of the rhizobia. As a result such products contained high levels of contaminant organisms that out compete slow growing bacterial inoculants (Deaker et al. 2004). A second step where problems frequently occur is during the application of the inoculant, either directly to the seed, or into the soil furrow immediately before sowing. Moist inoculant dusted onto seeds is easily dislodged during handling and sowing, to counter this adhesives are applied to seed to prevent such mechanical displacement. Appropriate storage of the inoculant is also essential, if it becomes dried out during storage its viability is seriously compromised or it is stored at too high a temperature. There is also evidence that seeds produce toxic exudates which can inhibit rhizobial inoculants applied to them (Deaker et al. 2004).

The success of commercial inoculants is dependent on the number of viable bacteria available to participate in the infection process at the point of use (Catroux et al. 2001). High numbers of rhizobia per seed typically increases nodulation, N_2 fixation and yield. For example, when inoculant concentrations on lupin seeds (*Lupinus* spp.) were raised from 2 cells to 1.86 x 10^6 cells per seed, the grain yield increased by 94% (Roughley et al. 1993). A similar positive response was demonstrated in clover (*Trifolium ambiguum* (M.) Bieb) where successful nodulation rates improved by over 60% when the number of rhizobia per seed was increased by an order of magnitude (Patrick and Lowther 1995). Such studies indicate that the size of the inoculum per seed is important and that it must be increased in response to environmental stress for successful nodulation to occur.

A number of countries have sought to improve inoculant quality through the application of legislation that defines the number of viable rhizobia per seed and acceptable levels of contamination (Stephens and Rask 2000). This has resulted in an increase in the quality of the inoculants produced (Lupwayi et al. 2000). However, a recent review highlighted that in many countries the quality of commercial inoculants remains poor and this appears to be exacerbated when regulation is voluntary or left to the discretion of the manufacturers (Catroux et al. 2001).

Stressful conditions in the soil

Many agricultural soils throughout the world are subject to one or more environmental stresses that inhibit the effectiveness of rhizobial-legume symbioses (Table 1). Over 20 years ago Amarger (1981) demonstrated that the pH of a soil could have a significant effect on the rhizobial community; under acidic conditions *Sinorhizobium meliloti* (the symbiont of alfalfa) was largely absent, whereas in alkaline soils *Bradyrhizobium* spp. that nodulate lupin could not be detected. These observations have significant repercussions for the use of N fixing bacteria when one considers that in Africa, Australia, Asia and the Americas, a large proportion of the soils used for cultivating crops are acidic (Date 2000). Moreover, agricultural practices can exacerbate this problem. For example, in Australia long term subterranean clover (*Trifolium subterraneum* L.) pastures have decreased the pH of already naturally acidic soils by 1 unit (Slattery et al. 2001). As pH decreases, toxic metal ions, particularly aluminium, also become soluble and this has resulted in significant decreases in productivity (Date 2000). Other heavy metals such as cadmium and zinc are also toxic to rhizobia and reduce N fixation in contaminated soils (Broos et al. 2005). The impact of alkaline soils on nodulation has been studied in the clover (*Trifolium alexandrinum* L.) inoculated with a commercial strain *R. leguminosarum* bv. *trifolii* TA1, that demonstrated indigenous field isolates out competed the inoculant for nodule occupancy (Denton et al. 2003)

Water availability can be a significant stress that restricts the cultivation of legumes in Mediterranean environments (Drevon et al. 2001; Mashhady et al. 1998). Inhibition of BNF is even more severe in the many agricultural soils that are subjected to prolonged drought, leading to the desiccation of soils, such arid or semi-arid land is common in sub-Saharan Africa (Shisanya 2002). Water scarcity is frequently countered by irrigation that in turn leads to soils becoming salinised, thus reducing BNF (Table 1) and water availability still further.

Drought often goes hand in hand with high temperatures, and in tropical regions it is common for the temperature to exceed the upper limit for both nodulation and N_2 fixation in legumes (Hungria and Vargas 2000). In contrast, at higher latitudes, low temperature and the length of the growing season can be inhibitory factors. In Northern Europe, chill tolerant varieties of white lupin (*Lupinus albus* L.) have been developed (Shield et al. 2000), however, the response of rhizobial inoculants to cold has received little attention. The sowing of legumes frequently occurs when temperatures in the soil are not optimal, this affects early root growth and decreases the survival and competitive advantage of inoculants added to the seed (Denton et al. 2003).

In the field, there will be a variety of stresses in a soil. For example, in Brazil, common bean is grown on 1.2 million hectares of land called the 'Cerrados'. The crop is subjected to temperatures in excess of 40^{o}C, water stress, soil acidity and aluminium toxicity. Indigenous rhizobial populations are low and inoculation with rhizobial strains required for adequate crop yields. However, commercial inoculants such as *R. leguminosarum* bv. *phaseoli* (SEMIA 4064) have been shown to lose their ability to fix N due to the extreme environmental stress in these soils (Hungria and Vargas 2000).

Competition with native rhizobia

Inoculation of legumes with rhizobia may be ineffective because it does not lead to nodulation. This is often explained as being indicative of the inoculants failing to compete with resident rhizobia. Theis et al. (1991) observed that as few as 10 indigenous rhizobia g^{-1} of soil effectively eliminated any response to inoculation. In regions where legume crops are indigenous, field strains of rhizobia can readily nodulate legumes although symbiotic effectiveness is often limited (Mhamdi et al. 2002). In contrast, in areas where legumes are exotic, such as Australia, the long term use of inoculants inevitably leads to introduced strains becoming naturalised in the soils. In South-Western Australia the pasture legume *Trifolium subterraneum* L. has been inoculated with *Rhizobium leguminsarum* bv. *trifolii* for many years. There are now naturalised populations of the bacterium in the soil that can compete for nodule occupancy with commercial strains but, in many cases, offer much less effective N_2 fixation (Collins et al. 2002). It has been frequently shown that indigenous or naturalised rhizobia are more saprophytically competent because they are better adapted to soil conditions than the commercial strains introduced as inoculants, particularly if there are stressful conditions in the soil (Slattery et al. 2004). For example, experiments in alkaline soils investigating the nodule occupancy of three clovers with *R. leguminosarum* bv. *trifolii* showed dominant isolates were alkaline resistant naturalised strains (Denton et al. 2002).

ENHANCING THE POTENTIAL OF BACTERIAL INOCULANTS

Bespoke Inocula

The most significant progress in improving the efficiency of inoculation as a boost to plant yield and soil N is to obtain high quality 'bespoke' rhizobial inoculants that are capable of tolerating and maintaining themselves within the specific soil environment to which they are applied. In order to obtain an effective inoculant, the selection of potential bacteria must be rigorous and the isolates must demonstrate the ability to;

- effectively nodulate and fix N,
- compete with indigenous rhizobia for nodule formation,
- tolerate soil conditions,
- survive in peat culture and on inoculated seed (Date 2000).

The saprophytic competence of inoculant strains is particularly important in marginal soils or those subjected to environmental stress. It is also essential that the bacteria employed are thoroughly tested to ensure that they offer the most effective means of plant growth promotion, as subsequent attempts to displace established populations of N_2 fixing bacteria with novel inocula are typically unsuccessful. Similarly inocula must be carefully matched to appropriate crop varieties to maximise the boost to yield and soil N. A particular problem is ensuring that selected strains do not compromise N_2 fixation in other agricultural crops grown in the same area (Howieson et al. 2000).

This overarching strategy has recently been articulated and demonstrated to produce successful results (Sessitsch et al. 2002 and references therein; Table 2). A good example is *Rhizobium tropici* PRF81, isolated from Brazilian 'Cerrados' soil, and recommended as a commercial inoculant for common bean (*Phaseolus vulgaris* L.) in Brazil since 1998 (Hungria et al. 2000). *R. tropici* has been shown to be more genetically stable than other common bean *Rhizobium* species under environmental stress (Flores et al. 1988) and, therefore, less likely to lose its symbiotic competence. Inoculation with PRF81 in a two year trial gave yield increases of up to 906 kg ha^{-1} compared to non-inoculated controls and yields which were not statistically different from those with 30 kg N ha^{-1} added. *R. tropici* PRF81 is now recommended as the inoculant of choice to Brazilian farmers (Mostasso et al. 2002). This success has encouraged a systematic search for more inoculants capable of forming effective symbiosis with the common bean in the stressful soils of the Cerrados. Five additional strains of indigenous rhizobia have been identified, with N fixing potentials equal or better than that of isolate PRF81. A similar approach has been adopted in Australia to try to obtain naturalised strains of rhizobia to utilise as alternatives to commercial inoculants. Work with Lucerne (*Medicago sativa* L.) found that rhizobia could be readily isolated from pastures with nitrogen fixing capacities comparable with commercial inoculants (Ballard et al. 2003). Similarly, indigenous *Bradyrhizobia* from Ghanaian soil were isolated and used to inoculate cowpea (*Vigna unguiculata* (L.) Walp.) giving yields comparable to plants fertilised with 70 kg N ha^{-1} (Fening and Danso 2002). Further examples of the exploitation of stress resistant and indigenous strains of rhizobia are given in Table 2.

Where developing highly specific inoculants is not cost effective, one solution is to develop 'elite' stress resistant inoculants, capable of stimulating BNF under a range of environmental stresses. Such a strategy can be an effective and economically viable compromise between the current approach of large scale 'elite' inocula production, which are often ineffective in marginal soils, and parochial isolates whose development as inocula may not be economically viable. For example, utilisation of acid tolerant *Medicago* cultivars and *Sinorhizobium* strains in acidic soils in South-Western Australia has been extremely successful in increasing herbage yield by 51% and seed by 31% (Howieson et al. 1991).

Searching for effective inoculants among indigenous rhizobia or in populations subjected to stresses and soil conditions similar to those where BNF is required has advantages over alternative strategies. Molecular biology, as a mechanism to improve strains of rhizobia used as inoculants, has not realised its potential because inoculants are produced and sold cheaply and therefore, do not merit the investment required to fully explore the effectiveness of genetic manipulation approaches. In addition, genetically modified bacteria need to be carefully evaluated to reassure regulators and the public of their safety before being widely disseminated in the environment. In contrast, naturally occurring rhizobia are both cheaper to develop and less problematical to utilise.

Table 2. Examples of studies exploiting rhizobial inoculants that are indigenous, or adapted to stressful soils which illustrate the actual or potential use of 'bespoke' inoculants. Commercial inoculant when included for comparative purposes are in bold.

Bacterium	Plant	Notes	Reference
Rhizobium tropici **CIAT899** Indigenous Rhizobia	*Phaseolus vulgaris* cv Coco	49 indigenous isolates from Tunisia and Morocco were found to be at least as efficient as commercial inoculant CIAT899 in symbiosis with local cultivar Coco.	Drevon et al. 2001
R. tropici **CIAT899** and PRF 81 Indigenous *R. tropici*	*Phaseolus vulgaris* L.	The Cerrados of Brazil is an area subject to high temperatures, low pH and water stress. Five indigenous *R. tropici* strains were shown in field trials, to be at least as effective than the commercial inoculant strains.	Mostasso et al. 2002
R. tropici PRF 35, 54 and 81	*Phaseolus vulgaris* L.	In field trial inoculation did not increase yield in first year compared to non-inoculated controls. In second year PRF81 did give significant increase in yield equivalent to 60 kg N ha^{-1} of inorganic fertiliser.	Hungria et al. 2000
R. tropici **CIAT899** *R. etli* (4 indigenous)	*Phaseolus vulgaris* L.	Field trial over 2 years gave increase equivalent to application of 50 kg ha^{-1} N in first year but no significant yield response in year 2. However, climatic conditions were unfavourable.	Aguilar et al. 2001
R. leguminosarum bv. *trifolii*	*Trifolium subterraneum* L.	Strain isolated from root nodules and tested for competitiveness in two Pakistani soils. In pot experiments, recovery of most capable strain from nodules was 100%.	Naeem et al. 2004
Rhizobium strains IRc1045 and IRc1050	*Leucaena leucocephala* (Lam de Wit.)	In a Nigerian study, *L. leucocephala* was inoculated in 1982 with *Rhizobium* spp resulting in 180 kg ha^{-1} N yr^{-1}. Ten years later uninoculated *L. leucocephala* fixed 150 kg ha^{-1} N yr-1. Serotyping indicated the nodules were predominantly occupied (96%) by IRc1045 and IRc1050. Both strains were originally isolated from Nigerian soils.	Sanginga et al. 1994
Sinorhizobium meliloti	*Medicago sativa* L.	Isolates obtained from salinised soil gave higher N_2 fixation than commercial isolates in salinised sand culture. However, inoculation of lucerne with these isolates in saline soil did not result in higher N_2 fixation as seen in sand culture.	Mashhady et al. 1998
Bradyrhizobium spp. (lupin)	*Lupinus albus*; *L. termis; L. triticale*	The ability of 6 strains on nodule number, mass and shoot and root dry matter accumulation in iron deficient alkaline soils was examined. 2 isolates increased all these parameters in contrast to the other 4 due to their ability to scavenge Fe through siderophore production.	Abd-Alla 1999
Bradyrhizobium japonicum WB108, WB112, WB1	*Glycine max* L. (Merr.)	In South African soils using 3 soya bean genotypes, 3 inoculants and 3 soil types, significant correlations were observed between amount of N fixed, soil type and inoculant, the seed protein content was significantly different depending on soil, genotype and inoculant.	van Jaarsveld et al. 2002
Bradyrhizobium japonicum UDDA 30, 31	*Glycine max* L. (Merr.)	Isolates were chosen as they were capable of growing in the cold (15ºC). In comparison with the most widely used commercial inoculant, these isolates gave increased nodule number, weight and shoot N yield.	Zhang et al. 2003

THE FUTURE OF BNF IN SUSTAINABLE AGRICULTURE

Agriculture has witnessed a decline in BNF based farming systems in recent years. This has arisen because of soil degradation, the development of herbicide resistant weeds and the arrival of new pathogens and pests. The forces now driving the evolution of legume based agricultural systems have rendered the crops traditionally obsolete used due to their susceptibility to disease and that the legumes and their symbionts are constrained by stressful soils (Howieson et al. 2000). As a consequence, there is a requirement to find a range of novel legumes that are less susceptible to disease and pests, can tolerate stressful soils and are deeper rooting to access water deep in the soil. Moreover, an increase in the diversity of the legumes exploited will provide more stability to these system overall. Hand in hand with the introduction of these legumes is the need to develop effective symbiotic partners matched to both host plant and soil conditions (Howieson et al. 2000). In developing countries we have discussed the issues surrounding the increased pressure on agricultural land to increase food production and the effect this is having in degrading the quality of many soils. If BNF is to remain a viable alternative to the use of inorganic N it must address these issues in order to generate a more effective, reliable and sustainable solution to the problem of N limitation in agricultural soils.

CONCLUSIONS

BNF has been proven to be an effective mechanism to enhance soil N. However, as we have seen expectations of the technology must be based on the rational use of diazotroph in an appropriate context. Free-living diazotrophs utilised as inoculants have still to convincingly demonstrate a reliable and long-term positive impact in boosting crop yield. While there is continued optimism that such bacteria may have role to play (Kennedy et al. 2004), many of the studies done to date suggest hormone effects on crop growth rather than furnishing them with additional N (Andrews et al. 2003). The use of symbiotic inoculants has a much more convincing track record, however, recent work in Australia (Howieson et al. 2000; Ridley et al. 2004) have demonstrated that problems are present even in agricultural systems where the use of BNF is well established. In the future the application of BNF must reflect a much more considered approach in which the cost benefit analysis takes into account the type of farming, the availability of suitable crops, the impact on sustainability within the system and most importantly the benefit for the farmer (Sessitsch et al. 2002). One element of this approach is to develop high quality inocula for use on specific crop cultivars optimised for the conditions within the soil. Here we have seen that the use of 'bespoke' inoculants developed from indigenous rhizobia offer the potential for high symbiotic and edaphic competence. Such bacteria should be more robust to soil stress and deliver a more reliable supply of fixed nitrogen to boost crop yield.

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