Use of specific N_2 fixing genotypes as crop inoculants: progress made and potential for stressful soil environments

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Abstract

Unlike N fertiliser, inoculation of crops with diazotrophic bacteria does not reliably increase crop yield. Inoculants added to plants are often ineffective due to an inappropriate mechanism of action that does not add substantial nitrogen to the system, low viability of the inoculant or poor persistence of the bacteria in the soil. In this paper we identify the major problems of using inoculants, and dicuss how improved effectiveness may be achieved by selecting strains of bacteria capable of tolerating soil stresses while effectively fixing nitrogen. The key elements of an effective inoculant are identified, as a mechanism that increases plant available nitrogen, a high quality inoculant appropriate to the crop cultivar being grown and an inoculant which is competitive and robust enough to tolerate the soil conditions it will encounter. We discuss the advantages of using specific N_2 fixing bacteria isolated from stressful soil environments as inoculants and illustrate, with examples from the literature, how such bacteria have been shown to provide effective inoculants for specific crops which are subsequently cultivated in those soils.

1 Introduction

The major nutrient limitation to crop production is the supply of nitrogen (N) available to the plants. The most consistently effective remedy for this is the application of inorganic nitrogenous fertilizer. Fertilizer application has allowed extremely intensive agricultural practices to be adopted. However, it has also led to an increase in environmental problems in particular, rapid eutrophication of

surface waters, increased atmospheric ammonia and nitrogen oxide concentrations and contamination of the groundwater with nitrates. Moreover, because of the cost of fertilizer, many poorer farmers and subsistance growers cannot afford to apply it. Such farmers typically cultivate more marginal land which is subject to degradation due to stresses such as drought, salinisation, erosion and nutrient depletion. Here, alternative strategies to fertiliser N have been sought to combat limiting soil N levels. Frequently, these make use of N_2 fixing legumes in rotations. Often, these crops are inoculated with N_2 fixing nodulating bacteria. Also, many free-living and endophytic N_2 fixing bacteria can interact positively with non-legume crops in ways which increase yields. A range of these bacteria have been isolated and used as inoculants of non-legume crops, in particular cereals, in attempts to maintain or increase productivity while reducing the need for N fertiliser. However, in order to be effective, such bacteria must be capable of surviving and forming effective partnerships with host plants despite the environmental stresses that they encounter.

In this review, we show the current progress and the potential of N_2 fixing bacterial inoculants to increase crop yield. Also, we highlight the problems which require resolving if the potential of this technology is to be realised. Firstly, we discuss the range of bacteria used as inoculants, focusing on the intimacy of the relationship with the plant and their mechanism of action. Secondly, we discuss the problems constraining the effectiveness of inoculation as an alternative to N fertiliser with emphasis on the use of rhizobial inoculants in the large number of agricultural soils subject to extreme environmental stresses. Inoculant persistence in the soil is a vital factor. However, persistence is frequently limited by; (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 we discuss current and potential approaches which may circumvent these problems. In particular, we advocate the use of native rhizobia as 'tailor made' inocula to provide crops in a particular soil type with a high quality, persistent and competitive inoculant.

2 Nitrogen fixing bacteria: intimacy of interaction with plant and mechanisms of action

Bacteria capable of fixing N are widely distributed in taxonomic terms, and the organisms which have been used as inoculants reflect this diversity. The free living bacteria often referred to as plant growth promoting rhizobacteria (PGPR) do not have an intimate symbiotic relationship with the crop on which they are inoculated. Many species of N_2 fixing bacteria such as *Azospirillum* species, pseudomonads and *Azotobacter* species are found living in the soil and rhizosphere. In some cases they are found in intercellular spaces within inner root tissues, as is observed with strains of *Azospirillum brasilense* inoculated onto wheat (*Triticum aestivum* L.) [1]. Such bacteria have been used extensively as inoculants of dryland graminaceous crops since the middle of the last century. Although many PGPR do have the capacity to fix atmospheric N, evidence from

large scale trials in Russia in the 1940s and 1950s with *Azotobacter chroococcum* on wheat and barley (*Hordeum vulgare* L.) and studies world wide in the 1980s with *Azospirillum brasilense* and *A. lipoferum* on a range of graminaceous crops indicates that there is no reliable positive effect on crop yield [2]. It appears that any additional N taken up by inoculated plants is primarily derived from the soil not N_2 fixation. The general opinion is that rhizosphere bacteria such as *Azotobacter* and *Azospirillum* act via changes in root morphology and physiology (probably hormone induced) which result in increased mineral nutrient and water uptake from the soil, especially during early growth: these effects result in greater crop growth and subsequently greater yield [2]. However, any effect is generally small, equivalent to that obtained by the application of around 30 kg N ha^{-1}. The N deficiency of a soil cannot be reliably countered by an inoculant which does not add substantial N to the system.

The N balance, ^{15}N isotope dilution and ^{15}N natural abundance studies provide strong evidence that some economically important tropical grasses including sugarcane (*Saccharum* spp.), kallar grass (*Leptochloa fusca* (L.) Kunth.) and wetland rice can obtain a substantial proportion of their N requirements from N_2 fixation [2]. There is also strong evidence that endophytic N_2 fixing bacteria play a major role in this process but that within plant species, the amount of N_2 fixed is at least partly dependent on plant genotype and geographical location [2, 3]. For example, in the case of sugarcane, so far only a few Brazilian varieties have been shown to definitely fix N, with a few studies from other counties producing equivocal or negative results using ^{15}N natural abundance. Although the specific micro-organisms responsible for this N_2 fixation have not been exclusively determined, it appears that *Azoarcus* spp. play the major role in N_2 fixation in kallar grass, while *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. could be important in N_2 fixation in sugar cane, however, many other as yet undiscovered bacteria are also likely to be involved [2, 4, 5]. It has been proposed that endophytic N_2 fixing bacteria have considerable potential for use in agriculture as the interior of the plant could provide a low O_2 environment necessary for the expression of nitrogenase [4]. In addition, endophytic bacteria will not have to compete with with rhizosphere bacteria for resources, and within the plant, transfer of C from the host to the bacteria and N from the bacteria to the host is likely to be more efficient [4]. However, if endophytic N_2 fixing bacteria are to be of use as inoculants, they must provide the plant with substantial N from N_2 fixation. Studies on the effects of inoculation of crops with N_2 fixing endophytic bacteria are at a preliminary stage and substantial experimentation is required before their potential can be adequately assessed.

The nodulating bacteria have been referred to as the *Rhizobiaceae*, and include several genera of Gram negative nodulating N fixing bacteria [6]. Recent revisions to the taxonomy of this group have shown that they can no longer be considered as a distinct family but are rather more widely dispersed throughout the order Rhizobiales of the Alphaproteobacteria. The most recent revision [7] has led to the proposal that the genus *Agrobacterium* be amalgamated with

Rhizobium. Many of these bacteria share the functional characteristics of nodulating and fixing N within their host plants, which include all the most widely exploited leguminous crops. Nodulation represents the most intimate relationship between plant and bacteria, and is capable of adjustment in terms of its anatomy and physiology in the face of environmental stress. Such responses are typically species specific [8].

Rhizobial inoculants have been shown to exert positive effects on crop growth through N fixation. However, the ability to form symbiotic N_2 fixing nodules cannot be relied upon to provide a positive impact on crop yield. In many cases, rhizobia may only poorly nodulate or form ineffective nodules on their host plant [9]. This is frequently because the inoculant are of low quality or are simply not competitive with indigenous rhizobia in the soil. However, rhizobial inoculants are a mature technology, subjected to wide scale and prolonged study. The body of literature on these inoculants provides the most complete information on the problems and potential of inoculants and will form the basis for the remainder of this review.

3 Constraints on the effectiveness of inoculants as an alternative to N fertiliser

The systematic use of N_2 fixing bacteria to enhance crop yield has been widely practised since early in the twentieth century. However, many of the current constraints on inoculation, namely, the poor quality of many inoculants, the lack of competitiveness of inoculants with indigenous rhizobia and the inability to tolerate environmental conditions in the soil have dogged the technology throughout its history.

3.1 Poor quality inoculant with low viability

Recent reports have cast doubt on the effectiveness of inoculation as a strategy to improve crop yield. This criticism has arisen because of the quality of inocula that is produced commercially. The estimate that 90% of inoculant has no practical effect on legume productivity [10] will clearly impact on the confidence placed in inoculation as a strategy for increasing crop yield. The low effectiveness of rhizobial inoculation is a function of rhizobial number. Low rhizobial numbers can arise because the bacteria in the inoculant have a low viability (low quality) or because, the bacteria used as the inoculant are not competitive with indigenous soil rhizobia and do not persist in the soil. The nodulation of a crop by a selected bacterial strain is a function of the number of viable bacteria available to participate in the infection process. High numbers of *Rhizobium* per seed typically increases nodulation, N_2 fixation and yield. For example, Roughley et al. [11] demonstrated that over a range of inoculant concentrations of 2 to 1.86 x 10^6 cells per seed, the dry matter and grain yield of lupin (*Lupinus* spp.) increased by 92% and 94%, respectively. This effect was also observed under environmental stress. For example, in acid soils, effective nodulation of caucasian clover (*Trifolium ambiguum* (M.) Bieb) increased from

5% to 66% as the number of rhizobia was increased from 0.2 x 10^3 to 2.63 x 10^3 per seed [12].

For adequate nodulation and increases in yield, the appropriate rhizobia must be present in high concentrations. In several countries the problem of inoculant quality has been addressed by governmental regulation defining the number of viable *Rhizobium* per seed and acceptable levels of contamination [13]. This has resulted in an increase in the quality of the inoculants produced [14, 15]. However, in many countries the regulation is voluntary or left to the discretion of the manufacturers and much poor quality inoculant is still being manufactured and distributed.

3.2 Competition with native rhizobia

Inoculants may be ineffective because they do not form nodules with the crop. This is often explained as being indicative of the inoculants failing to compete with resident rhizobia. Theis *et al.* [16] observed that as few as 10 indigenous rhizobia g^{-1} of soil effectively eliminated any response to inoculation. The most straightforward solution to poor nodulation in particular environments by 'elite' inocula is to isolate indigenous rhizobia from the soil to use as an inoculant. Any such indigenous rhizobia must be an effective symbiotic partner of the crop genotype to be used, they must remain viable in the inoculant carrier and be genetically stable. A good example where this approach has been successful 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 [17]. 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. Also, *R. tropici* is more genetically stable than other common bean *Rhizobium* species under environmental stress [18]. Therefore, rhizobia indigenous to the soil or isolated from wild legumes represent a pool of largely unexplored biodiversity [19], which have potential to be exploited as inoculants for crop legumes.

3.3 Stressful conditions in the soil

The failure of inoculants to produce effective nodulation can also arise because the bacteria fail to cope with the environmental conditions in the soil to which they are applied. Many agricultural soils throughout the world are subject to environmental stress of one or several kinds. In Africa, Asia and South and Central America, around 70% of the soils used for crops are acidic [20]. Around 90 million hectares in Australia are also naturally acidic, and agricultural practices are further increasing acidity. For example, continuous subterranean clover (*Trifolium subterraneum* L.) pastures have decreased the pH by 1 unit [21] giving significant decreases in productivity. As pH decreases, toxic metal ions, particularly Al^{3+}, become soluble. Increased Al^{3+} reduces the availability of calcium and phosphorus resulting in the inhibition of nodulation [20]. Water

availability is also a significant stress in many soils. This is often exacerbated by prolonged irrigation which ultimately leads to soils becoming salinised, thus reducing water availability still further. Such soils are widespread and restrict the cultivation of legumes across the Mediterranean basin and the Middle East [22, 23]. In addition, salinised soils have a cost burden for their reclamation. For example, in Australia, remediating saline soils has been estimated to cost 220 million dollars annually [24]. Other areas are subject to prolonged drought, which can lead to dessication of soils. Arid or semi-arid land such as this is common in sub-Saharan Africa, and an increase in food production in these areas is required to feed the growing population [25]. Frequently, drought is accompanied by high temperatures. In tropical soils, the soil temperature can be higher than 40 °C at 5 cm depth which exceeds the upper temperature limits for N fixation in tropical legumes. There is also inhibition of nodulation at such temperatures [26]. In contrast, at higher latitudes, low temperature can be the inhibitory factor. In Northern Europe, chill tolerant varieties of white lupin (*Lupinus albus* L.) have been developed [27], however, the response of rhizobial inoculants to cold has received little attention.

In some cases, a variety of stresses are present in a soil. For example, in Brazil, the Cerrados, which make up 25% of the land area, are subjected to temperatures in excess of 40 °C, water stress and soil acidity with resulting Al toxicity. Common bean is grown on 1.2 million hectares of this land, therefore, inoculants tolerant of the conditions are required to boost yields. 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 [26]. This has catalysed the search for novel indigenous rhizobia which resulted in the success of *R. tropici* PRF81, discussed above. A continuing program of common bean inoculant selection has identified five other strains of indigenous rhizobia, with commercial potential. In comparative trials against the currently recommended commercial bean inoculants (including PRF81), the five strains employed were as effective as PRF81 in nodulating and improving the yield of the crop over a two year trial [28]. 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} [29].

4 Situations where inoculation is successful: opportunities for 'tailor made' inoculants

Despite the problems associated with the application of inoculants to leguminous crops, there are particular circumstances when inoculation does have clear benefits. Inoculants applied to the seed or soil are particularly effective in marginal soils that have a low natural population of N-fixing bacteria and limited N. The Cerrados of Brazil is an example of how common bean yields can be improved by an appropriate inoculant such as PRF 81. Similarly in sub-Saharan Africa, inoculation of soybean (*Glycine max* (L.) Merrill) with *Bradyrhizobium japonicum* increased yield from 500 to 1500 Kg ha^{-1} [30].

The introduction of leguminous crops into a novel geographic area can also benefit from the application of an inoculant as the soils may not contain appropriate rhizobial populations. For example, utilisation of acid tolerant *Medicago* cultivars and *Sinorhizobium* strains in southwestern Australia has been extremely successful in increasing herbage yield by 51% and seed by 31% [31]. The most significant progress in improving the efficiency of inoculation as a boost to plant yield and soil N is to obtain high quality inocula which are capable of tolerating and maintaining themselves within the soil environment in which they are used. Often, as discussed above, such organisms are native rhizobia which are often more effective at nodulating legumes than exotic strains introduced as inoculants. The future on inoculation may be in preparing 'tailor made' inocula adapted to the soils in which they are to be used and matched to the specific crop genotype to be grown.

4.1 Criteria for developing 'tailor-made' inoculants

In order to obtain an effective inoculant, the selection of potential bacteria must be rigorous. The strain selected must fulfil certain criteria in order to be effective as an inoculant. The selection of appropriate strains must address the requirement that the isolate has the ability to effectively nodulate and fix N, that it can compete with indigenous rhizobia for nodule formation and can tolerate soil conditions. In addition, the isolate must be able to survive in peat culture and inoculated seed [20]. The saprophytic competence of inoculant strains is also an important consideration in areas subjected to environmental stress. Typically in such marginal soils where the economic margins are smaller, the additional cost of inoculation may not be offset by increases in yield [32]. Therefore, rotations involving legumes requiring routine inoculation may not be economically viable. 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.

Pursuing this line of reasoning to its logical conclusion would result in each specific soil having its own specific inoculum. Clearly in terms of time and effort this would not be economically viable in marginal soils. However, developing 'elite' stress resistant inoculants, capable, for instance, of being effective in a range of acidic soils may 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, soil surveys in S.E. Australia have indicated that a lack of effective rhizobia may be limiting the performance of annual medics (*Medicago* spp.). *Rhizobium* populations are high in such soils but do not nodulate effectively [21]. Introducing effective acid tolerant rhizobia, which are selected to be effective symbionts of the medics could provide a solution to this problem.

The approach of 'bioprospecting' for novel inoculants could have advantages over alternative strategies. In the last decade, molecular biology was touted as a mechanism to improve strains of rhizobia used as inoculants [33]. This potential has not been realised because the cost benefit analysis is not favourable. Inocula 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.

5 Conclusions

I. Current inoculants have not been proved to be a reliable alternative to N fertiliser in improving crop yields. This is frequently due to the mechanism of action of inoculants. It is stressed that the N deficiency of a soil cannot be reliably countered by an inoculant which does not add substantial N to the system.

II. Inoculant production must have rigorous quality control procedures to ensure that the product is of high quality.

III. New inoculants must be thoroughly tested to ensure they are appropriate to the crop being sown, competitive in the soil with indigenous rhizobia and robust enough to maintain genetic stability and ecological competitiveness when subjected to the environmental stresses they will encounter in the soil.

IV. Indigenous rhizobia from soils subjected to environmental stress offer an opportunity to obtain competitive, pre-adapted inoculants to increase crop yield in marginal soils.

References

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Section 12
Brownfields