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Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between *Azospirillum* and wheat

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Abstract

Recent advances towards achieving significant nitrogen fixation by diazotrophs in symbioses with cereals are reviewed, referring to the literature on the evolution of effective symbioses involving rhizobia and *Frankia* as microsymbionts. Data indicating that strains of *Acetobacter* and *Herbaspirillum* colonizing specific cultivars of sugarcane as endophytes make a significant contribution to the nitrogen economy of this crop improves the prospects that similar associative systems may be developed for other gramineous species such as rice and wheat. By contrast, the transfer of nodulation genes similar to those in legumes or *Parasponia* to achieve nodulation in crops like rice and wheat is considered to be a more ambitious and distant goal. Progress in developing an effective associative system for cereals has been materially assisted by the development of genetic tools based on the application of *lacZ* and *gusA* fusions with the promoters of genes associated with nitrogen fixation. These reporter genes have provided clear evidence that 'crack-entry' at the points of emergence of lateral roots or of 2,4-D induced *para*-nodules is the most significant route of endophytic colonization. Furthermore, using the laboratory model of *para*-nodulated wheat, there is now evidence that the ability of azospirilla and other nitrogen fixing bacteria to colonize extensively as endophytes can be genetically controlled. The most successful strain of *Azospirillum brasilense* (Sp7-S) for endophytic colonization and nitrogen fixation in wheat seedlings is a mutant with reduced exopolysaccharide production. Most other strains of azospirilla do not colonize as endophytes and it is concluded that though these are poorly adapted to providing nitrogen for the host plant, they are well adapted for survival and persistence in soil. A research program combining the study of endophytic colonization by azospirilla with an examination of the factors controlling the effectiveness of association (oxygen tolerance and nitrogen transfer) is now being pursued. It is proposed that a process of facilitated evolution of *para*-nodulated wheat involving the stepwise genetic improvement of both the prospective microsymbionts and the cereal host will eventually lead to effective nitrogen-fixing associations. In the attempt to achieve this goal, continued study of the endophytes occurring naturally in sugar cane and other grasses (e.g. *Azoarcus* sp.) should be of assistance.

Introduction

Globally, biological N_2 fixation, as a proportion of the total input of nitrogen to support the growth of crops, has probably been declining. It appears that a significant reduction in the relative use of fertiliser-N can only be achieved if biological nitrogen fixation is made directly available to cereal crops in an effective associative system with some of the characteristics of the legume symbiosis. Experience since the plant-associating diazotrophs were discovered by Döbereiner and her coworkers (see Baldani et al., 1983) has shown that this objective will be elusive and that achievement of nitrogen fixation with cereals is still some distance away although there may be other benefits from inoculation of field crops (Okon and Labandera-Gonzolez, 1994). Despite this, the probability of eventual success of nitrogen fixation with

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cereals should now be regarded as significant. Success will require a period of intensive developmental research, seeking a better understanding of the nature of plant-microbial associations while overcoming the obstacles to an effective symbiosis in a progressive fashion.

The thesis to be developed in this paper is that achieving this ambitious goal using microsymbionts such as *Azospirillum* may demand a process of *facilitated evolution*, designed to ultimately yield a spontaneously effective symbiosis. An essential set of desirable genetic characteristics need to be identified and then selected in new combinations of existing microbial and plant germ plasm or by deletions of genes that currently prevent desirable outcomes. In this review paper, it is intended to test this goal against recent research findings in the literature and to demonstrate that progress towards the goal has already been achieved.

Evolution of nitrogen-fixing symbiosis

In considering the possible evolution of a nitrogen-fixing symbiosis for cereals it is worth reviewing the information available regarding the evolution of the successful symbioses involving rhizobia (including bradyrhizobia and azorhizobia) and *Frankia* as microsymbionts.

Symbiosis may be defined as a mutual interdependence of dissimilar organisms. Its main characteristic is the complementary nature of the metabolic apparatus of each of the partners so that the overall task of satisfying nutritional needs for growth and reproduction are shared by both organisms. Although discussion of the conditions leading to the successful evolution of nitrogen fixing symbioses tend to be rather speculative it is significant that many species are involved as partners in the nitrogen-fixing symbioses. The apparent range of species involved is so diverse that several independent developments of nitrogen-fixing symbiosis in legumes have been proposed (Provorov, 1994). The advantage to the host plant of symbiotic nitrogen fixation is obvious but Udvardi and Kahn (1993) point to the key fact that the rhizobia have evolved a genetic regulatory system that requires nitrogen fixation to occur without associated ammonia assimilation in the bacteroids. The interactive signal transduction pathway induces nitrogenase in response to low oxygen concentration, even when bacterial cells have enough nitrogen. They pose and attempt to answer the question of what the bacteroids gain by fixing nitrogen if they are not nitrogen-starved. However, attempting to assess the value of fixing nitrogen to bacteroids may be unnecessary in considering the phenomenon of symbiosis. Probably of much more significance to the rhizobia in terms of evolutionary selection pressure is the enhanced ability to eventually increase cell numbers and therefore to persist in soil.

Another hypothesis suggests (Sprent, 1994) that two separate nodulation events occurred in the humid tropics during the evolution of legumes in the late Cretaceous period. One of these involved an ancestor of *Rhizobium* and entry by root infection. This was initially parasitic and provided little benefit until bacteria were released from infection threads as in modern crop species. The other concerned a photosynthetic ancestor of *Bradyrhizobium* using a wound infection on stems, but never involved infection threads. The need for tolerance to stress by bacterial strains would also impose evolutionary constraints, where not all rhizobia (e.g. in arid regions) are capable of symbiosis, since symbiotic genes may be an expensive encumbrance in these conditions. Lateral transfer of material on megaplasmids could lead to a wide range of symbiotic and non-symbiotic forms in response to local pressures. When environmental constraints are superimposed on initial evolutionary developments, the result is an apparently chaotic situation where there is no obvious pattern of co-evolution between hosts and rhizobia. Evidence of such coevolution may be amenable to molecular analysis. Direct evidence of such interactive coevolution of host plant and microsymbiont was presented by Devine (1988) in the case of soybeans related to the production of rhizobitoxine by bradyrhizobia that can potentially interfere with chlorophyll synthesis leading to chlorotic plants.

Apart from the symbioses involving nodulated legumes (plus the non-legume *Parasponia*) and the rhizobia, a diverse range of non-legumes form nodular symbioses with *Frankia.* The taxonomic scheme of Cronquist (1981) classified plants with root nodule symbioses so that host plants of rhizobial symbionts were placed in subclasses Rosidae and Hamamelidae and those of *Frankia* in the widespread subclasses of Rosidae, Hamamelidae, Magnoliidae and Dilleniidae.

More recently, this broad phylogenetic distribution of nodulated plants has been challenged and angiosperm phylogenies based on DNA sequence comparisons reveals a more coherent group than previously thought (Chase et al., 1993; Soltis et al., 1995). This molecular data now indicates a single origin of the

predisposition for root nodule symbiosis and also supports the occurrence of multiple origins of symbiosis within this more restricted group (Soltis et al., 1995; Swensen and Mullin, 1997). These findings indicate that only one small group of angiosperms in a single clade possesses the ability to host nitrogen-fixing microsymbionts.

There is still sufficient plant and microbial diversity involved to surmise that the initiation of nitrogen-fixing symbiosis was not an absolutely unique event. Possibly, some plant species or their ancestors may have periodically lost and then regained a symbiotic state in response to altered selection pressures. The complexity of the molecular dialogue now recognised as controlling the establishment of many legume-*Rhizobium* symbioses presumably represents a refinement of simpler relationships existing formerly. However, no mechanism can readily be proposed that would allow a sequential development of such a complex interactive process without initial advantages such as mutually beneficial nitrogen fixation. Thus we may assume that even legume symbiosis involved a prototype stage of development involving fewer genes but of lower stability. Alternatively, advantages such as improved mineral nutrition, stimulation of plant root growth or mild parasitism may have provided initial mutual benefits favouring acceptance of microbes by the plant host, with insertion of nitrogen fixation at a later stage. The genetic significance of the host plant in evolving an effective nitrogen-fixing symbiosis is obvious, supported by direct experimental evidence of heritable genetic variation for nitrogen fixation in pasture (Pinchbeck et al., 1980) and grain legumes (Smartt, 1986).

The reasons that cereals and most other Gramineae provide little clear evidence of effective symbioses with nitrogen-fixing microorganisms bear examination. The cereals do not fall within the clade of nodulating angiosperms mentioned above and it can be surmised that nodulation of a similar kind to that involving rhizobia and *Frankia* might be more difficult than previously thought (Swensen and Mullin, 1997). Therefore, a less developed nitrogen-fixing association between bacteria and Graminae might be be a preferable goal at this stage. Possibly, the relatively low nitrogen content of annual cereals does not provide sufficiently strong sink strength for nitrogen to elicit evolution involving as many genes as found in legume symbiosis. Although modern cereals bred for much higher yield than their genetic ancestors probably require more nitrogen, the deliberate choice of fertile soil or its supplementation with fertiliser-N may have minimised selection pressures for developing or stabilizing nitrogen-fixing associations in cereal crops. For this reason, the search for germ plasm to facilitate nitrogen fixation in cereals should not ignore primitive cereals. The fact that cereals require much less nitrogen than legumes both simplifies the task in terms of the rates of nitrogen fixation required but possibly destabilizes any putative symbiosis that may develop since the needs for nitrogen may be more readily met from the soil.

Since our review of biological nitrogen fixation in non-leguminous field crops that included a discussion of *para*-nodulation (Kennedy and Tchan, 1992) considerable advances in knowledge have occurred. It is also striking that the focus is now on several nitrogen-fixing bacteria paid little attention before 1990. For example, strong credence is now granted to the significance of the naturally-occurring nitrogen-fixing association between sugarcane and *Acetobacter diazotrophicus* discussed below. Several other bacterial species potentially capable of nitrogen fixation with cereals are receiving more attention, including *Herbaspirillum* sp. and *Azoarcus* sp. Verification that some of these bacteria carry out symbiotic nitrogen fixation with crop plants and were already bringing previously unrecognised benefits to agricultural production is providing a welcome stimulus to attempts to engineer nitrogen fixation for the growth of wheat and rice. The availability of a range of genetic tools and reporter genes since 1992 is also providing strong impetus to the search for new information of value for this objective.

Models for development of N_2 fixing symbiosis in cereals

Key factors in establishing effective symbioses with cereals

Kennedy and Tchan (1992) discussed the key issues and problems likely to be encountered in any program of research seeking nitrogen fixation in cereals. In summary, these issues included:

1. the importance of adequate colonization and the probable ineffectiveness of diazotrophs located at the root surface (rhizoplane) in achieving significant nitrogen fixation. The main reasons for this included the inadequacy of carbon substrates diffusing from the root surface and competition

for these substrates from other microbes. Quispel (1991), in a discussion of the topic of achieving nitrogen fixation in cereals, drew attention to the probable significance of endophytic diazotrophs located within the root system in achieving such an objective. Recent research data to be described in this paper emphasises the validity of his proposal.

2. the oxygen paradox, posed by the fact that this gas is simultaneously an essential terminal electron acceptor for respiratory synthesis of ATP yet is extremely toxic to the nitrogenase enzyme. A large part of the physiological and morphological character of leguminous and non-leguminous nodules is devoted to regulating oxygen pressure within a satisfactory range.
3. the effective transfer of nitrogen to the host plant. This will be discussed later, but as indicated above in the discussion of evolution in legumes, the efficient transfer of newly-fixed nitrogen is one of the most salient features of the legume symbiosis. Even in the case of symbioses based on rhizobia and *Frankia*, there is obviously much to learn about the regulation of this process.

These issues regarding carbon substrates, oxygen and nitrogen transfer remain of central importance. However, as a result of new discoveries with both naturally occurring systems and with laboratory models, progress in our understanding of the solutions required is better defined. This review paper will illustrate this conclusion using some natural systems and then by focussing on the performance of a various diazotrophs in *para*-nodulated wheat.

Sugar cane

^{15}N and nitrogen balance studies conducted recently (see Boddey et al., 1995 for review) have shown that certain Brazilian cultivars of sugar cane can obtain over half their needs for nitrogen from BNF (>150 kg N ha^{-1} $year^{-1}$). The discovery of endophytic N_2-fixing bacteria within the roots, shoots and leaves of sugar cane is suggestive that these organisms are responsible, and that earlier scepticism about these claims was misplaced. It is suggested that the fact that sugar cane in Brazil has been bred over a long period for high yields with low fertilizer-N inputs has favoured the emergence of this naturally occurring symbiosis.

The key bacteria recognised so far in N_2-fixing sugar cane are *Acetobacter diazotrophicus* (Cavalcante and Döbereiner, 1988; Reis et al., 1994) and *Herbaspirillum* spp. (Baldani et al., 1986). Strains of *Herbaspirillum* also infect gramineous crops including rice in both the roots and the aerial tissue. *A. diazotrophicus* is a Gram-negative rod forming a rising pellicle in N-free medium with 100 g $L^{-1}$of sucrose. It is claimed to show a higher oxygen tolerance than *Azospirillum* species, continuing to fix up to 4 kPa (Reis et al.,1990). Its demonstrated capacity to directly transfer half the nitrogen fixed to an amylolytic yeast (*Lypomyces kononenkoae*) in mixed culture (Cojho et al., 1993) suggests that *A. diazotrophicus* would be capable of a similar transfer of fixed nitrogen to the plant tissues in sugar cane. Apparently, the high sugar requirement of this organism prevents it being found in significant numbers in soil but it has been recovered from a few other plant species. Studies on infection and colonization of micropropagated sugar cane seedlings using immunogold labelling indicated that it favoured crevices associated with lateral roots (James et al.,1994) and it is suggested that the bacteria are able to migrate within the xylem stream to the tops of the plants.

H. seropedicae with several other species have been isolated from sugarcane roots, stems and leaves but this species survives poorly in soil (Baldani et al., 1992). However, sorghum seedlings were able to stimulate growth of residual organisms in soil so that increased numbers of bacteria could be observed in the rhizosphere and roots (Olivares et al., 1993).

Döbereiner (1996) suggests that this organism is an obligate endophyte and that in sugar cane, it could be capable of conducting a complementary metabolism with *A. diazotrophicus* by consuming organic acids formed by the latter from sugar, thus maintaining homeostasis for pH within the plant tissues.

For future research with cereals, it will be of interest to obtain definition of the following aspects of this symbiosis:

1. The processes of infection and initial colonization of plants and the cell numbers per unit of host plant tissue needed for effective nitrogen fixation. Current data infers that the whole plant may be colonized by the nitrogen-fixing microsymbionts. If so, this raises some interesting questions regarding protection from oxygen produced in leaves, provision of carbon substrates at many such sites and the ability of bacteria and plant cells to achieve symbiosis.
2. The possible advantages of mixed communities of endophytic diazotrophs in achieving effective nitrogen fixation.

3. The robustness of the system under conditions of adequate nitrogen from soil. It is suggested that fertilizer nitrogen acts to severely reduce the ability of these diazotrophs to carry on nitrogen fixation. It will be important to establish the degree to which the system can tolerate periodic availability of fixed nitrogen and whether recovery of nitrogen-fixing ability can be achieved when required.

Azoarcus *spp. as nitrogen-fixing endophytes*

Originally isolated from Kallar grass (*Leptochloa fusa* Kunth) growing in saline-sodic soils of Pakistan, a strain of the gram-negative nitrogen-fixing bacterium *Azoarcus* sp. BH72 has been described (Reinhold-Hurek et al., 1993) that also colonizes grasses such as rice in laboratory experiments (Hurek et al., 1994). *Azoarcus indigens* is the type species and a second named species, *Azoarcus communis*, includes a strain obtained from French refinery oily sludge (Reinhold-Hurek et al., 1993). Bacteria of this genus have a strictly aerobic type of metabolism, fixed nitrogen microaerobically, and grew well on salts of organic acids but not on carbohydrates; the five species have now been identified as belonging to the beta subgroup of Proteobacteria (Hurek and Reinhold-Hurek, 1995). Related toluene-degrading bacterial isolates were investigated by 16S rRNA sequence analysis and a study of their biochemical and physiological features made (Zhou et al., 1995). Phylogenetic trees were constructed from 16S rRNA sequences and showed the isolates as belonging to a phylogenetically coherent cluster representing a sister lineage to *Azoarcus* species. A new species of the nitrogen-fixing genus *Azoarcus* was named *Azoarcus tolulyticus.*

A thorough and in-depth study of *Azoarcus* as an endophyte is yielding potential benefits, particularly the observation of extremely active form of nitrogenase activity associated with a characteristic morphology of the cells. Such high nitrogenase expression may be important in obtaining adequate rates of nitrogen fixation in associative systems with limited numbers of bacterial cells and the phenomenon deserves further study.

Ultrastructural analysis of cells in the course of hyperinduction revealed that complex stacks of intracytoplasmic membranes called diazosomes are formed; these are absent under standard nitrogen-fixing conditions (Hurek et al., 1995). The iron protein of nitrogenase was highly enriched on these membranes, as evidenced by immunohistochemical studies. Diazosome deficiency in NifH/K- mutants, a deletion mutant in the *nifK* gene and the character of NH_4^+-grown cells suggested, in concert with the membrane localization of nitrogenase, that these structures are specialized membranes related to nitrogen fixation. In batch cultures of *Azoarcus* sp. strain BH72, at nanomolar oxygen concentrations in the presence of proline, cells can shift into a state of higher activity and respiratory efficiency of nitrogen fixation in which diazosomes related to nitrogen fixation are formed (Karg and Reinhold-Hurek, 1996). Induction of intracytoplasmic membranes was most pronounced when *Azoarcus* sp. strain BH72 was cultured with an ascomycete originating from the same host plant, Kallar grass.

The invasive properties of *Azoarcus* sp. strain BH72, the endorhizospheric isolate of Kallar grass, on gnotobiotically grown seedlings of rice cv. IR36 and *L. fusca* were studied (Hurek et al., 1994). To facilitate localization and to assure identity of bacteria, genetically engineered microorganisms expressing β-glucuronidase were also used as inocula. β-glucuronidase staining indicated that the apical region of the root behind the meristem was the most intensively colonized and light and electron microscopy showed that strain BH72 penetrated the rhizoplane preferentially in the zones of elongation and differentiation and colonized the root interior both inter- and intracellularly. In addition to the root cortex, bacteria were also found in the xylem. No evidence was found for *Azoarcus* residing in living plant cells; plant cells were apparently destroyed after bacteria had penetrated the cell wall. A common pathogenicity test developed for tobacco leaves indicated that representative strains of *Azoarcus* spp. are not phytopathogenic. Compared with the non-inoculated controls, inoculation with strain BH72 significantly promoted growth of rice seedlings. However, this effect was reversed when the plant medium was supplemented with malate (0.2 g L^{-1}). Nitrogen fixation was apparently not involved, because the same response was obtained with a *nifK* mutant of strain BH72 with a Nif^- phenotype. PCR and Western immunoblotting, using primers specific for eubacteria and antibodies recognizing type-specific antigens, respectively, indicated that strain BH72 could colonize rice plants systemically. When this data is considered with the observation that *Azoarcus indigens* expresses *nifH* and acetylene reduction activity to a maximum oxygen tension of 6.5% (Vande-Broek et al., 1996), this genus as a potential endophytic nitrogen-fixing microsymbiont for cereals merits continued study.

Hypertrophies on rice roots and their colonization by microbes

Recently, de Bruijn et al. (1995) have reviewed the potential and pitfalls of extending symbiotic interactions between nitrogen-fixing organisms and cereals such as rice. They considered chemical signalling between plant and microbe, nodulation and the prospects for symbiotic nitrogen fixation with such systems and the reader is advised to consult this critical review for this discussion which will not be covered here. The results of experiments carried out in China on the induction of "nodule-like" structures on rice roots by rhizobia were highlighted and data on attempts to extend these experiments in the USA given, providing some additional understanding of the induction of these structures by rhizobia and possibilities for their colonization.

They concluded that the formation of hypertrophies on rice roots infected and colonized with microbes had been confimed, but that little evidence supported their designation as "nodules" or even nodule-like. The frequency of formation of the root structures was low, but non-saprophytic colonization of the rice-root endorhizosphere had clearly been observed and deserved further study in this important area of research.

Rhizobia and azorhizobia as inoculants for cereals

There are potentially many approaches to the development of symbioses in cereals. The legume-*Rhizobium* or *Frankia* root nodule symbioses may be used directly as models, seeking to identify key features that would be regarded as essential for the development of analogous systems in cereals. The earlier approach used by Cocking and associates with wild *Parasponia* rhizobia and wheat and the recognition of a moderate degree of intercellular and intracellular infection associated with shortened, thickened lateral roots (Cocking et al., 1992) could be recognised as seeking a more primitive stage of legume nodule development, following their earlier work on more direct methods of inducing nodular structures with enzymes. More recently, this group has focussed their attention on associations between azorhizobia and cereals, also studied with some success by Chen et al. (1992), seeking evidence of possible chemical signalling between the bacteria and host plants that could influence success in establishing successful symbiosis based on colonization associated with the points of emergence of lateral roots (Webster et al., 1997). Using a *lacZ* marker gene, they showed that the flavanone naringenin at 10^{-5} *M* stimulated significantly the colonization of lateral root cracks and intercellular colonization of wheat roots by azorhizobia. Our experience with *Azorhizobium caulinodans* in *para*-nodulated wheat has been described (Kennedy et al., 1997; Yu and Kennedy, 1995). Acetylene reduction rates with *A. caulinodans* were found to be much less than with a strain of *Azospirillum brasilense* - mainly as a result of the small proportion of the *para*-nodules that were colonised (Yu and Kennedy, 1995). Recently Sabry et al. (1997) showed that wheat grown in pots and inoculated repeatedly with *A. caulinodans* colonised root tissues at the points of emergence of lateral roots and appeared to contribute significant amounts of nitrogen fixation to the plant. Acetylene reduction rates were similar to those reported with *para*-nodulated wheat. A potential advantage of *A. caulinodans* as an endophyte is that, like *Azospirillum*, it can more readily express nitrogenase activity than other members of the Rhizobiaceae which almost generally only fix nitrogen in legume nodules.

The para*-nodule induced with synthetic auxins such as 2,4-D*

During the past four years since we reviewed the status of biological nitrogen fixation in non-leguminous field crops (Kennedy and Tchan, 1992), several papers (see Katupitiya et al., 1995a, 1995b; Kennedy, 1994; Yu and Kennedy, 1995) have described our development of the laboratory model of associative N_2 fixation known as the *para*-nodule. The approach is based on the initial observations of Yanfu Nie at Shandong University that 2,4-D acting as an auxin could stimulate colonization by rhizobia in 'nodular structures' modified from the lateral roots of many plant species. We have emphasized that these structures, derived from the induction of the initials of lateral roots, are quite dissimilar to legume nodules particularly when colonized by azospirilla. In this case, the bacteria colonize intercellularly almost exclusively, usually in the basal zone of the *para*-nodules where plant cells appear loosely packed. Even when extensively infected with rhizobia (Nie et al., 1992), there is no evidence of intracellular colonization of living wheat root cells as occurs in legume nodules.

Although the use of synthetic auxin such as 2,4-D presents difficulties for field application, we consider that the use of this procedure is justified as a laboratory model, providing rates of improved coloniza-

tion and nitrogenfixation that allow the controlling factors to be studied. It may prove possible to provide similar outcomes to the use of 2,4-D biologically, such as by using mutants producing higher amounts of indoleacetic acid. However, it should be recognised that many systems of agricultural monoculture already apply very stringent chemical treatments, sometimes with dramatic effects on plant development (e.g. cotton production using hormones and chemical defoliants).

We have not so far sought to demonstrate that *para*-nodulated wheat can fix nitrogen sufficiently well to become independent of soil nitrogen, preferring to concentrate on the factors controlling colonization in preliminary work. However, with improved experimental techniques we have now obtained enrichments using $^{15}N_2$ much higher than those reported previously (Yu et al., 1993), around 0.5 atom % ^{15}N in root total-N for overnight exposures, suggestive that most of the approximately 10^7 azospirilla in the root system of wheat seedlings (per 100 mg fresh weight) are actively fixing nitrogen (Wood and Kennedy, in preparation; see Kennedy et al., 1997).

Of extreme importance in this recent research has been the use of genetic markers and reporter genes based on fusions of the promoters of *nif* genes of *Azospirillum brasilense* with *lacZ* (Liang et al., 1991) and their application to the study of the colonization of wheat roots by azospirilla in collaborative work between our two laboratories (Arsène et al., 1994). These fusions have the facility to allow both visual and enzymic detection of bacterial cells in association with wheat. It is probable that analysis of β-galactosidase activity in the case of constitutively expressed fusions provides a better estimate of bacterial cell numbers than most probable number dilution counts. Similar genetic tools for the study of colonization of cereal roots by azospirilla based on the use of *gusA* fusions are also being applied for studies with cereals (Vande-Broek et al., 1993, 1996). These genetic tools provide much more reliability both in the data obtained and more confidence in its interpretation (see also Stoltzfus et al., 1997).

Key factors in colonization of para-nodules by *A. brasilense*

Our recent research using the genetic tools with *para*-nodulated wheat has led to the recognition of a number of factors that facilitate improved N_2 fixation under our specific conditions of assay.

Endophytic colonization by the flocculation$^-$ mutant of A. brasilense *Sp7*

A fortunate spontaneous mutation in our laboratory strain of *A. brasilense* Sp7, resulted in the selection of a new strain (Sp7-S) showing inability of colonies to bind Congo red with the loss of flocculating ability (Katupitiya et al., 1995b) from lowered exopolysaccharide (EPS) production and reduced swarming (Pereg et al., 1996). This reduction in EPS production was accompanied by loss of the ability of the bacterial cells to adhere to the wheat root surface when seedlings were inoculated. At the same time as the loss of EPS production there was an increased ability of Sp7-S to colonise wheat roots internally (endophytically), in an intercellular fashion, particularly in crevices at the point of emergence of lateral roots and in the basal region of the *para*-nodules (auxin-modified lateral roots). A simple interpretation is that the absence of EPS production provided a smooth-surfaced bacterial cell with less ability to adhere to the root cell surface of plants. The 'stickiness' of the wild-type Sp7 may tend to trap these cells on root surfaces and inhibits 'crack-entry' of cells into crevices at the root surface and near the points of emergence of lateral roots or *para*-nodules. The presence of such points of entry in the loosely structured monocots such as wheat was pointed out previously (Kennedy and Tchan, 1992). The associated qualities of absence of encystment and retention of the vegetative phenotype by Sp7-S both in pure culture and on wheat roots also seems likely to be of importance.

Upon prolonged incubation in semisolid or solid media many isolates of azospirilla form ovoid cysts which are distinct from the typical vibrioid or S-shaped cell morphology of this genus (Lamm and Neyra, 1981). Cyst-enriched cultures are much more resistant to desiccation than normal vegetative cultures (Lamm and Neyra, 1981; Papen and Werner, 1982) and their formation is presumed to represent a mechanism by which azospirilla can persist in soil during unfavourable environmental conditions. Encystment of *A. brasilense* Sp7 (ATCC 29145) is favoured in medium containing fructose (8 m*M*) and KNO_3 (0.5 m*M*) forming thick-walled structures filled with poly-β-hydroxybutyrate and associated with the production of a melanin-like pigment in aged cultures (Sadasivan and Neyra, 1987). Cyst formation in broth cultures

is associated with flocculation and exopolysaccharide formation (Sadasivan and Neyra, 1985).

We have consistently observed apparent encystment of Sp7 on the wheat root surface after several days of colonization, shown by the ovoid shape of the cells (see Figure 1A); this observation contrasts with the consistently vibrioid shape of cells of Sp7-S when colonizing wheat seedlings (Katupitiya et al., 1995b), remaining in the vegetative form under all conditions we have observed on wheat roots. We have also found using *lacZ* and *gusA* fusions with Sp7-S and Sp7 simultaneously that colonization occurs preferentially at the same locations as observed when they colonize wheat separately (Katupitiya et al., 1997). Papen and Werner (1982) showed the inhibition of nitrogenase activity occurred when *A. brasilense* was encysted in broth culture so we assume that the cyst-like cells of Sp7 on the root surface could also be nitrogenase deficient although we have no direct demonstration of this. However, even these cyst-like cells still exhibit expression of *nifH* activity as the blue colour of β-galactosidase activity (Arsène et al., 1994; Figure 2), possibly indicative of a residual expression of nitrogenase activity, consistent with the lower activity acetylene reduction activity observed (Katupitiya et al., 1995b).

Colonization, carbon substrates and oxygen conditions suitable for nitrogenase expression in 2,4-D treated wheat seedlings

The effect of 2,4-D treatment on wheat seedlings is complex. Lateral root initials are activated *en masse*, providing many foci for endophytic infection of roots by 'crack-entry'. However, following initiation these lateral root initials fail to extend, thus providing the rounded nodular structures. This effect is concentration dependent, *para*-nodules being formed at a concentration of 2,4-D well below that at which herbicidal effects are exerted. Synthetic auxin also induces increased concentrations of soluble sugars (Feng, et al., 1997) and amino acids (Yu and Kennedy, unpublished). A glucose-utilizing mutant of *A. brasilense* Sp7-S (Sp7-Sg) had a modified pattern of colonization of wheat roots (Feng et al., 1997), with more bacteria occurring in intercellular channels established between rows of cortical cells. However, we have no evidence that this mutant is more capable of nitrogen fixation. In attempting to match the needs for carbon substrates by potential endophytes to those available in the plant it should be considered that cereals like wheat and rice are C_3 plants operating a Calvin photosynthetic cycle rather than the C_4 Hatch-Slack cycle involving organic acids as intermediates (Kennedy, 1992) that occurs in sugar cane.

Evidence has been obtained using *nifH-lacZ* that the FlcA$^-$ mutant (Sp7-S) when colonising *para*-nodulated wheat roots (Deaker and Kennedy, 1996) shows greater β-galactosidase activity over a higher range of oxygen concentration (Figure 2), consistent with the endophytic mode of colonization by Sp7-S shown in Figure 1B. This result for *nifH* expression is in close agreement with the rates of acetylene reduction observed previously in *para*-nodulated wheat (Kennedy and Tchan, 1992), indicating that Sp7-S is more protected from the effects of oxygen than the Sp7 wild-type when colonizing the roots of wheat seedlings. Experiments using *nifH-lacZ* to monitor suitable oxygen conditions for nitrogen fixation with *para*-nodulated wheat seedlings growing in soils of different bulk density are currently in progress.

Ammonia excreting mutants

Previous attention has been paid to the selection of ammonia-excreting mutants of azospirilla as having potential benefits in associations with plants (Machado et al., 1991). Christiansen-Weniger (1992) employed an ammonia-excreting of *A. brasilense* in studies on *para*-nodulated wheat, but the degree to which this strain can function as an endophyte has not been determined. More recently, Christiansen-Weniger and Vanderleyden (1994) described the colonization of *para*-nodulated maize roots by the same ammonia-excreting mutant of *Azospirillum*, labelled with a *gus* fusion.

A double mutant of Sp7 selected to colonize endophytically and to facilitate ammonia transfer (Sp7-SA) is currently being studied in our laboratory to determine whether there can be a significant benefit from the association between *Azospirillum* and wheat (Wood and Kennedy, 1996). This research (Wood, Ritchie and Kennedy, unpublished) aims to establish the mechanism of ammonia excretion (see Figure 3) and its relationship with membrane potential, relating the potential to excrete ammonia to the physiological conditions actually existing in wheat roots.

Genetic control of EPS production and flocculating ability

The recognition using *lacZ* markers that the spontaneous mutant Sp7-S presented a distinctly different

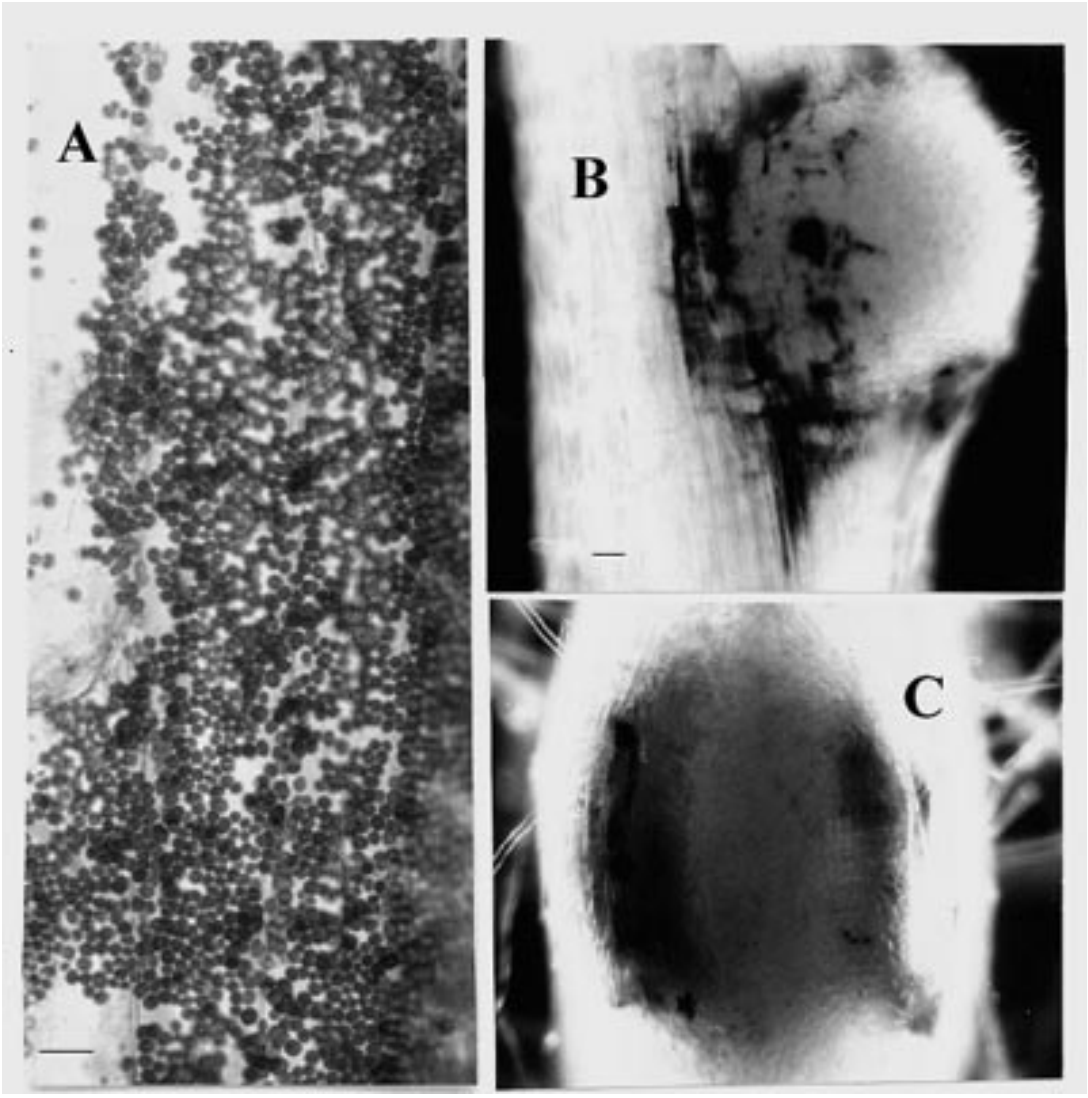

Figure 1. **A**) Cysts of *Azospirillum brasilense* Sp7 *nifA-lacZ* fusions at the rhizoplane of *para*-nodulated wheat seedlings. Plants were grown hydroponically for 10 days after inoculation (Zeman et al., 1992) and stained with X-gal (Arsène et al., 1994). *Bar* = 5 μm. **B**) Endophytic *A. brasilense* Sp7-S-*nifA-lacZ* fusions colonizing *para*-nodules on wheat seedlings stained with X-gal at 10 days growth after inoculation and treatment with 2,4-D. Cells of Sp7-S and Sp7-SA (ammonia-excreting mutant) with a FlcA$^-$ phenotype (failing to bind Congo red and to flocculate and encyst in broth) are only found in crevices on the root surface, at the points of emergence of lateral roots and in the basal zone of *para*-nodules as shown in the photograph. **C**) Endophytic *Herbaspirillum seropedicae* (ATCC 35892) *nifA-lacZ* fusions stained with X-gal have a similar pattern of colonization as *A. brasilense* Sp7-S. *Bar* (B and C) = 50 μm.

phenotype to that of Sp7 on wheat roots that also profoundly affected the colonization pattern observed opened the way to genetic analysis of this character. It was found possible to complement Sp7-S with DNA fragments from a gene bank derived from the Sp7 wild-type held by C. Elmerich at the Institut Pasteur, restoring the property of binding of Congo red by colonies on agar plates and flocculation in liquid culture (Katupitiya et al., 1995b). Subsequent studies (Pereg et al., 1995) have shown that site directed Tn5 insertions in the complementing region of the chromosome of Sp7 produced mutants that did not flocculate or bind Congo red and that could be complemented by a shorter segment of DNA derived from that described previously (Katupitiya et al., 1995b) for a gene involved in the regulation of the ability to flocculate designated *flcA*

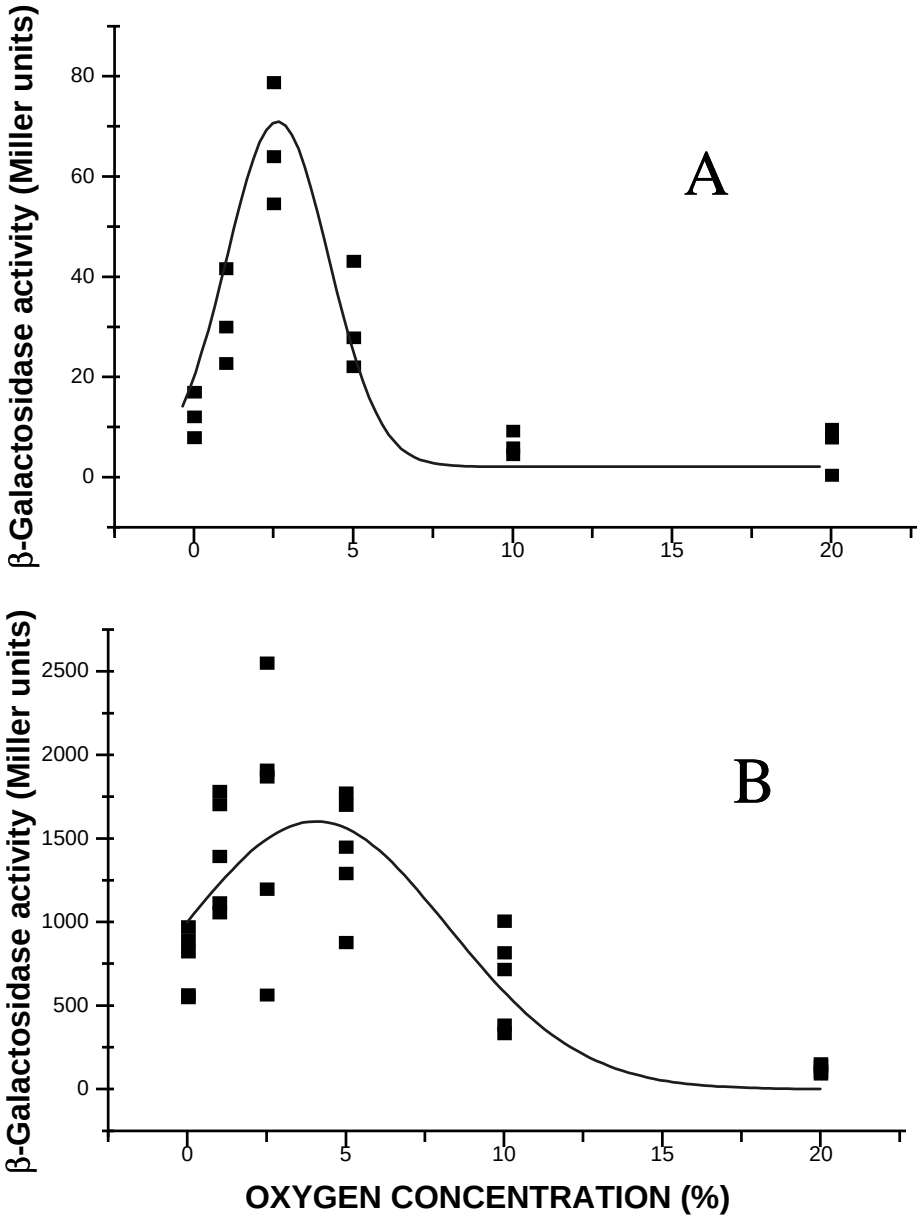

Figure 2. *nifH-lacZ* expression on *para*-nodulated wheat by *A. brasilense* Sp7 (**A**) and Sp7-S (**B**). The β-galactosidase activity (Miller Units per mg root protein) for separate seedlings is shown, following overnight incubation of seedlings at the oxygen pressure shown. Wheat seedlings were previously grown for several days in air-saturated hydroponic solution, completely repressing *nifH-lacZ* expression (Deaker and Kennedy, 1996 and in preparation).

(Pereg-Gerk et al., 1997). These Tn*5-flcA*$^{-}$ mutants also had reduced surface colonization of wheat roots, similar to Sp7-S, but it was observed that Sp7-S lacked the ability to swarm, probably as a result of the absence of lateral flagellae while the Tn5 mutants retained the ability to swarm (Pereg et al., 1996). Thus the difference in the phenotype for colonization between Sp7 and Sp7-S involves more than one mutation. Sp7-S is still motile and thus probably retains its polar flagellae. Subsequently, the *flcA* gene has been identified, its nucleotide sequence established and the regulation of the gene studied (Pereg et al., 1996, 1997).

The origin of the original stable mutant Sp7-S is uncertain, but it may have originated from deliberate

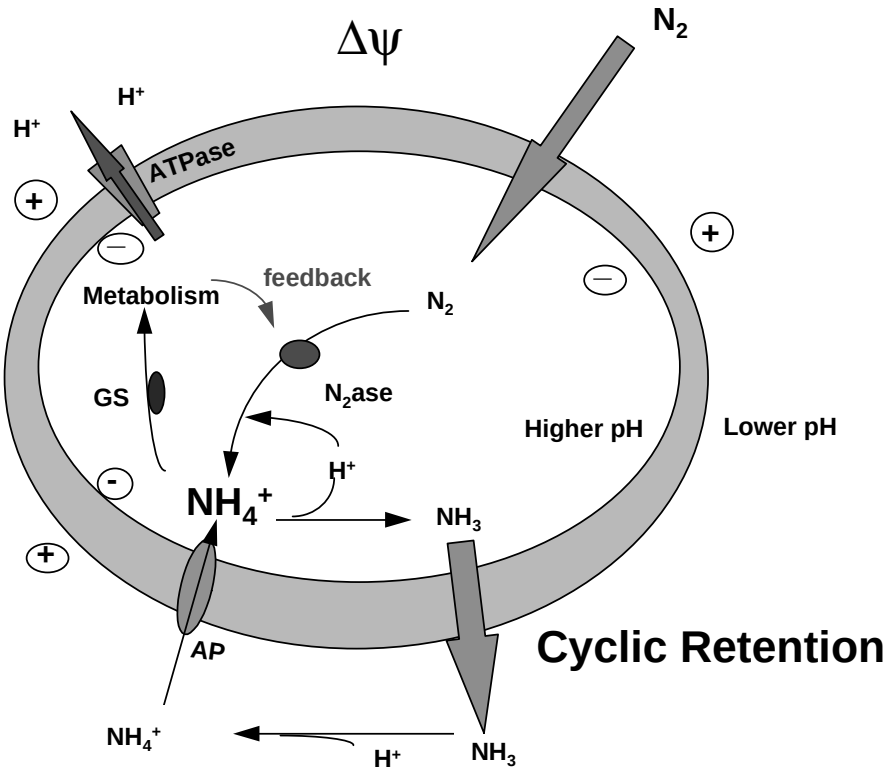

Figure 3. Model for nitrogen transfer from diazotroph to host plant. The hypothesis of cyclic retention (Kleiner, 1985) proposes that ammonia is normally retained within bacterial cells for assimilation following nitrogen fixation by an effective transport mechanism (e.g. ammonia permease, AP). In mutants, a lesion in AP, or in the ammonia-assimilating enzymes (glutamine synthetase, GS or glutamate synthase) causing high internal concentrations of ammonia, can lead to leakiness. The mutant *A. brasilense* Sp7-SA has a lesion in GS expression (Wood and Kennedy, 1996) and the influence of membrane potential ($\Delta\Psi$) on possible ammonia leakage is being studied (Wood, Ritchie and Kennedy, unpublished).

attempts by Anita Zeman in our laboratory to count and isolate endophytic cells of *A. brasilense* using a culture of Sp7 from the CSIRO (Division of Plant Industry, Canberra, Australian Capital Territory) collection obtained by Y T Tchan from the late Dr Alan Gibson around 1989. A second example of the Canberra Sp7 culture obtained subsequently by the senior author from Dr Gibson was dissimilar to Sp7-S, binding Congo red and flocculating in broth, but still appeared intermediate in other phenotypic properties between Sp7 from the Institut Pasteur collection of C. Elmerich and Sp7-S, indicating that Sp7 (Paris) and Sp7-S differ in more than one gene. Possibly, sterilisation of the root surface using brief treatments with mercuric chloride or ethanol (Sriskandarajah et al., 1993; Zeman et al., 1992) preferentially selected Sp7-S since it is predominantly endophytic in 2,4-D treated wheat seedlings.

Subsequent work on the isolation of spontaneous mutants of Sp7 forming colonies that fail to bind Congo red and flocculate has shown that such mutations are relatively frequent. Some of these mutations proved unstable (L Pereg-Gerk, unpublished), but no instances of Sp7-S losing the mutant character has been observed during five years of laboratory culture.

It is noteworthy that the original source of both these cultures of Sp7 (Canberra and Paris) was directly from Johanna Döbereiner in Brazil, probably in the late 1970s. Obviously, a degree of evolutionary drift of genotypes has occurred. On the other hand, the significant phenotypic differences in colonization of wheat roots that have resulted produced fortunate results in our laboratory.

Performance of other nitrogen-fixing bacteria in para-*nodules*

It was of interest to determine the ability of different nitrogen-fixing bacteria to colonize wheat seedlings

Table 1. Colonization pattern and acetylene reduction activity by different strains of diazotrophs in *para*-nodulated wheat

Strain	Predominant colonization mode	Acetylene reduction assay	Oxygen sensitivity
A. brasilense Sp7	Rhizoplane	+	High
A. brasilense Sp7-S	Endophytic	+++++++	Medium
A. brasilense Sp7-SA	Endophytic	+++++++	Medium
A. lipoferum	Rhizoplane	++++	Medium
Herbaspirillum seropedicae	Endophytic	++++++	Medium
Acetobacter diazotrophicus	Rhizoplane	-	-
Derxia gummosa	Rhizoplane	++	Low
Azotobacter vinelandii	Rhizoplane	+++++	Low
Rhizobium spp.	Endophytic	-	-

Wheat seedlings were treated with 2,4-D and inoculated with diazotrophs as previously described (Zeman et al., 1992). Colonization mode was determined by light microscopy using *nifA-lacZ* transconjugants stained with X-gal (Arsène et al., 1994).

(Kennedy et al., 1997). The performance of seven different bacterial strains is summarized in Table 1. As shown in the table, only *A. brasilense* Sp7-S, *A. brasilense* Sp7-SA (an ammonia-excreting mutant isolated in this laboratory by C. Woods) and *Herbaspirillum seropedicae* performed as endophytes in a manner meeting the criteria for significant nitrogen fixation. The colonization pattern of *H. seropedicae* (ATCC 35892) on wheat seedlings is similar to that of *A. brasilense* Sp7-S (see Figure 1b) , the *flcA*$^{-}$ mutant. According to Döbereiner (1996), *Herbaspirillum* is an obligate endophyte and is not usually found in the exorhizosphere. This is borne out by our experiments using *lacZ* transconjugants (Kennedy et al., 1997). It will be of interest to see whether the EPS composition of its surface is similar to that observed with the Sp7-S mutant.

To identify associative nitrogen fixers that possess a more oxygen-tolerant nitrogen-fixation system, Vande-Broek et al. (1996) analyzed the expression of an *Azospirillum brasilense nifH-gusA* fusion and the acetylene reduction activity as a function of the oxygen concentration in eight aerobic associative diazotrophs (*Acetobacter diazotrophicus, Alcaligenes faecalis, Azoarcus indigens, Azorhizophilus paspali, Azospirillum brasilense, Azospirillum irakense, Burkholderia vietnamiensis* and *Herbaspirillum seropedicae)*. Based on maximum oxygen concentration for activation of the *nifH* fusion and acetylene reduction, these organisms were classified into three groups of increasing oxygen tolerance. The groups were: (i) *Acetobacter diazotrophicus, Alcaligenes faecalis, Azospirillum brasilense, Azospirillum irakense, Burkholderia vietnamiensis* and *Herbaspirillum seropedicae* (maximum oxygen tension for acetylene reduction between 2.0 and 3.0%); (ii) *Azoarcus indigens* (maximum oxygen tension for acetylene reduction 6.5%); and (iii) *Azorhizophilus paspali* (maximum oxygen tension for acetylene reduction at 8.5%).

However, our results with different strains of *Azospirillum* and other organisms such as *Derxia gummosa* indicates that the pattern of colonization of wheat seedlings and the degree of endophytic character may be of more significance than the inherent tolerance to oxygen. No doubt the availability of reporter genes such as *nifH-gusA* and *nifH-lacZ* will greatly facilitate future work designed to show the the effectiveness of colonization of cereal roots by diazotrophs.

Conclusion

The ambitious goal of achieving nitrogen fixation by cereals naturally arouses controversy. However, despite the biological obstacles, there are strong and obvious grounds for making an attempt to achieve it, based on environmental and economic considerations. This goal is in keeping with the trend towards a more organically based agriculture and would be welcomed by many sympathetic to this movement and by the large number of subsistence farmers world-wide for whom fertilizer costs are prohibitive.

It must be recognized that feeding the increasing world's population in the last two decades has only been possible because of a huge expansion in the application of nitrogenous fertilizers and the extra pro-

duction from biological nitrogen fixation has probably been minimal. Some may even question the need for new systems capable of nitrogen fixation and there is no doubt that fertilizer-N must remain of predominant importance for the time being. In our opinion, the need for sustainable nitrogen-fixing systems is sufficiently great that there is an obligation on scientists to take some prudent risks in setting research goals.

We stress that even though the probability of success of obtaining an effective associative system with wheat soon is low (probably less than 0.5), this probability in the longer term is likely to approach unity. The progress since 1992 discussed in this short review suggests that the research is on schedule to deliver positive outcomes in the medium term of 5-15 years. This is particularly so if success is regarded as including reliable gains in crop production from associations with diazotrophs also involving other interactive factors influencing the nutrition and biological health of field crops.

Further development of the *para*-nodule so that it may be prepared for performance trials under field conditions is now our immediate goal. At this stage, however, the use of synthetic auxins and *para*-nodulation should be regarded as providing a re-iterative laboratory model for selecting desirable genetic features in azospirilla. Currently, there are challenges with respect to factors such as whether the high numbers of azospirilla found endophytically in wheat seedlings (ca. 10^8 cells g^{-1} fresh weight roots) can be maintained in larger plants and whether nitrogenase activity will be adequate with the oxygen concentrations found in soil. It seems likely that overcoming these obstacles will require significant genetic improvements of both host plant and bacteria as further steps in the process of facilitated evolution. However, these improvements may be hastened by selections made *in planta* aimed at generating superior genotypes favouring endophytic colonization of the plant.

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