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## Nodulation in Cereals as a Means to Decreasing Their Dependence on Nitrogenous Fertilizers – An Achievable Target or a Dogma

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**Abstract:** Nitrogen is the most common nutrient element, limiting crop production. Introduction and use of chemical N fertilizers has therefore led to a significant increase in crop yields. A major proportion of the nitrogenous fertilizers used in agriculture is applied to staple cereals like rice, wheat and maize. However, because of inherent economical and environmental implications of fertilizer N use, efforts have consistently been made to induce/enhance biological nitrogen fixation in cereals. Induction of nodulation has therefore been the main target of researchers over the past few years. This paper presents an evaluation of the achievements and prospects of nodulation in cereals (with particular reference to wheat and rice) as a means to decrease their dependence on fertilizer N.

**Key words:** Legume, paranodules, wheat, rice, 2,4-D, nitrogen fixation

### Introduction

Nitrogen is the major component of atmospheric gases (ca 78%) and represents 99.96% of the total that is found on earth, in the sea and around. Of the remaining 0.04%, biosphere contains only 0.005%. In spite of the small proportion found in living beings, N is most often the limiting nutrient for crop production since only a fraction of atmospheric  $N_2$  is made available to the plants through biological nitrogen fixation. Introduction and use of chemical nitrogenous fertilizers has therefore resulted in substantial increases in crop yields. About 42 million tons of fertilizer N is being used annually on a global scale for the production of 3 major cereal crops i.e., wheat, rice, and maize (17, 9 and 16 million tons, respectively). However, the crop plants are able to use about 50% of the applied fertilizer N, while 25% is lost from the soil-plant system through leaching, volatilization, and denitrification etc. causing an annual financial loss of ca 3 billion US\$. Incidentally, the losses are similar for the 3 crops mentioned above but twice as much from rice compared with wheat and maize when computed on the basis of per unit of fertilizer N applied.

The loss of fertilizer N is of concern not only because of economic reasons but also due to the pollution potential of different N forms. Some of the adverse environmental effects of excessive use of nitrogenous fertilizers include i) methemoglobinemia in infants due to  $NO_3$  and  $NO_2$  in waters and food, ii) cancer due to secondary amines, iii) respiratory illness due to  $NO_3$ , aerosols,  $NO_2$  and  $HNO_3$ , iv) eutrophication due to N in surface waters, v) material and ecosystem damage due to  $HNO_3$  in rain water, vi) plant toxicity due to high levels of  $NO_2$  and  $NH_4$  in soils, vii) excessive plant growth due to more available N; depletion of stratospheric ozone due to NO and  $N_2O$ . It is because of these concerns that concerted efforts have been made to reduce the use or increase the efficiency of fertilizer N uptake by crop plants.

Although multiple choice is available for enhancing use efficiency of fertilizer N and decrease its losses, making the cereal crops (major users of fertilizer N) relatively self sufficient in their N demands has been a persistent dream of researchers over many decades. For this purpose, leguminous crops have always fascinated the scientists as a model for developing the same or a similar N acquisition system in cereals. According to Kennedy *et al.* (1997), a significant reduction in the relative use of fertilizer N can be achieved if atmospheric N is made available to cereals directly through an effective associative system with some of the characteristics of legume symbiosis. This is because of the demonstrated/estimated potential of legumes to add atmospheric N to the natural and artificial ecosystems. Of a total of 139 million tons biologically fixed N added to the system annually, 25% comes from legumes i.e., 35 million tons which is only slightly less than that supplied to agroecosystems through chemical fertilizers. This figure demonstrates the significance of legumes in agricultural

and natural N cycles. Most of the legumes meet up to > 70% of their N demands through this process and may fix 57 to 600 kg  $N\ yr^{-1}\ ha^{-1}$ , minimum being for soybean (57 - 94 kg  $N\ yr^{-1}\ ha^{-1}$ ) and maximum (128-600 kg  $N\ yr^{-1}\ ha^{-1}$ ) for alfalfa. However, N economy of legume system in terms of N loss may not be significantly different from that of non-legume crops. A review of literature will demonstrate a loss of 18 and 20% of the plant N from legume and cereal crops, respectively. Thus, extending  $N_2$  fixation to cereal crops may prove economically feasible but not necessarily an environment friendly proposition. Nevertheless, economic reasons alone should be sufficient to instigate the scientists finding ways of extending biological nitrogen fixation to cereals. This paper presents an analysis of the prospects/extent of inducing nodulation in wheat and rice. Earlier, an analysis of the nature of legume bacterium association has been reported (Azam, 2001).

**Why to induce nodulation in wheat and rice?:** The efforts to make cereal crops, especially wheat and rice, relatively self reliant in nitrogen demands are desirable because of i) heavy requirements for nitrogenous fertilizers which are manufactured from non-renewable energy resources, ii) economic reasons, especially in developing countries iii) low efficiency of fertilizer N use by plants, and iv) environmental pollution potential of nitrogenous fertilizers. Global agriculture relies heavily on nitrogenous fertilizers with an annual cost of more than 45 billion US\$ and consuming substantial proportion of fossil fuels (Ladha *et al.*, 1997). Wheat and rice, which occupy an area of 370 million hectares worldwide with a grain yield of 1200 million tons, consume 62% (26 million tons) of nitrogenous fertilizers. Wheat is able to use 30-50% of the applied fertilizer N depending upon the variety and fertilizer management. A significant proportion (20-30%) is lost from the soil-plant system through a) volatilization from soil and plant, b) denitrification, and/or c)  $NO_3$  leaching. Rice is still more inefficient in fertilizer N use and losses as high as 70% of the applied fertilizer N could occur mainly through denitrification. Low use efficiency and heavy losses of fertilizer N not only affect agricultural economy, but the later has serious environmental implications as well (Boumann, 1996, Smith *et al.*, 1997). In economic terms, an increase of 1% in fertilizer efficiency is estimated to save more than US\$ 234 million in fertilizer costs worldwide (Raum and Johnson, 1999).

Conversely, this could be achieved through biological nitrogen fixation. Multi-disciplinary approaches have been identified and put to practice over the past few decades to increase use efficiency and decrease the losses of fertilizer N. These include a) breeding crop varieties with higher fertilizer use efficiency, b) management of fertilizer N, c) use of chemicals specific for different N transformation processes e.g., urease inhibitors to slow the process of  $NH_4$  formation from urea, which is the most common

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N fertilizer and nitrification inhibitor to regulate the process of  $\text{NO}_3$  formation, d) supplementation/integration of fertilizer N with organic manures e.g., green manures. These approaches applied singly or in combination, have certainly helped alleviate the problems arising as a result of fertilizer N use. Fertilizer management through improved formulations, mode and time of application, and placement etc. has been found helpful to overcome some of the problems. Likewise, a large variety of chemicals has been tested as potent inhibitors of specific N transformation processes including urea hydrolysis and nitrification. Using these inhibitors, not only use efficiency of fertilizer N is enhanced, but also a significant reduction occurs in losses due to  $\text{NH}_3$  volatilization, denitrification and  $\text{NO}_3$  leaching. Currently, increasing attention is being paid to the development of controlled-release and stabilized fertilizers that seem more efficient and environmental friendly (Trenkel, 1997).

Manures especially green manures have been found as a good supplement or replacement to fertilizer N in addition to being soil conditioner for sustainable soil productivity. Because of the ability to fix atmospheric N through symbiosis with rhizobia, leguminous crops have been the most favourite for green manuring. Some of these crops may add up to  $400 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ . However, their N supplying potential is generally over-emphasized, as only 20 to 30 % of the residue N is generally available for crop uptake (Azam, 1990). Nevertheless, the overall beneficial effects of green manures on soil fertility are quite well established.

In spite of above, extending the process of biological  $\text{N}_2$  fixation to wheat and rice has been of immense research interest probably due to the fact that nitrogen derived from fixation is considered more economical and environment friendly. This consideration seems logical because the  $\text{N}_2$  fixing plant systems do not require fossil fuel for their N supply and the fixed N is not as susceptible to losses as the fertilizer N. On a global level, annual contribution of BNF is estimated at 139 million tons. Of this, 25% comes from fixation by grain legumes (35 mT), 29% from forests and woodlands, 32% from permanent grasslands, 7% from non-legumes, and 7% from unused land. For free-living microorganisms e.g., blue green algae, *Azotobacter* and *Clostridium* the values are 25, 0.3 and  $0.1\text{--}0.5 \text{ kg ha}^{-1} \text{ yr}^{-1}$ , respectively. Of the non-symbiotic relationships, *Paspalum* adds 20 kg, *Brachiaria* 40 kg, kallar grass 90–180 kg, sugarcane up to 200 kg, and rice 6 kg of  $\text{N ha}^{-1} \text{ yr}^{-1}$ . These amounts, although variable provide a good proposition of extending biological  $\text{N}_2$  fixation to non-legumes particularly the cereals.

**Induction of nodule-like structures in cereals and their homology to legume nodules:** Legume nodule is a modified lateral root in terms of structure and development. Its homology with lateral roots has been inferred by a comparison with other nitrogen-fixing nodules, especially those formed on actinorhizal plants in response to *Frankia* inoculation or on *Parasponia* roots following inoculation with *Bradyrhizobium*. Cell differentiation as well as cell proliferation involved in nodule formation is different from that in lateral root development (Denarie and Cullimore, 1993). The most distinct change associated with the formation of effective legume nodule is the presence of leghaemoglobin, an oxygen binding protein considered necessary for effective  $\text{N}_2$  fixation. However,  $\text{N}_2$  fixation by *Rhizobium* strains can occur even in the absence of leghaemoglobin, while the later may be induced under anaerobic conditions in a number of plant tissues other than nodules (Jacobson-Lyon *et al.*, 1995).

Identification of *nod* factor (the end product of the expression of *Rhizobium meliloti* Nod genes) has been one of the most exciting findings (Lerouge *et al.*, 1990) in the studies of legume nodulation. These factors are N-fatty acid oligoglucosamines that are commonly found in the members of family Rhizobiaceae. *Nod* factor is reported to elicit root deformation as well as cortical cell division that lead to the formation of nodule primordium. Sprent and de Faire (1989) emphasized that many of the widely accepted dogmas for "normal" symbioses, e.g., root hair infection and the necessity of the bacteria to be released from infection threads before they differentiate into  $\text{N}_2$  fixing forms, are not universal.

Infection through wounds is now well established as a normal part of nodule initiation, so that the idea of a fixed, obligatory procedure cannot be sustained any further. Infection threads develop following damage to cells, and the damage could be caused in any manner. Despite genetic studies lasting more than a decade, no evidence has been found that the legume-rhizobium symbiosis involves unique plant gene products that are necessarily restricted to plants that are reported to successfully nodulate and fix  $\text{N}_2$ . Neither does any of the proposed steps in this symbiosis offer a uniqueness that would absolutely exclude different plant species allowing establishment of persistent symbioses with diazotrophs.

Plant growth hormones or phytohormones have indeed been found to play a role akin to *nod* factors in inducing root modifications that lead to the development of nodules (Hirsch *et al.*, 1997). 2,4-D, a commonly used herbicide having an auxin-like action is reported to induce effective nodulation in otherwise non-nodulating soybean (Akao *et al.*, 1991). This finding suggests a damaging effect of 2,4-D on plant roots that may serve as a prelude to microbial infestation of roots. Apparently, 2,4-D removes one of the barriers to nodulation and facilitates the entry of the bacterium into the roots. An effect similar to that of 2,4-D, has been obtained by using cell wall degrading enzymes. Cellulase and pectolyase are reported to facilitate the entry of  $\text{N}_2$  fixing microorganisms into the roots of many legumes and non-legumes including cereals (Cocking *et al.*, 1990). This would mean that a sufficiently damaging substance is required for malformation or so-called nodulation followed by bacterial entry into the wound, where it may or may not work depending upon the preferences and capabilities of the plants. It is quite likely, however, that chemicals like enzymes and phytohormones having cell-wall damaging properties could be used to induce nodulation in non-legumes as well as cereals. In legumes, Iannetta *et al.* (1997) suggested that species of rhizobia have evolved towards symbiotic status by development of cell-wall degrading enzymes on 2 levels, firstly to stop or limit secretion and/or synthesis of wall-degrading enzymes capable of eliciting host plant defense reaction, and secondly to express high levels of enzymes stimulated by the presence of compatible host tissue polysaccharides or polysaccharide containing moieties. The cereals appear to have developed a stronger defense mechanism against such an invasion.

In non-leguminous dicots and cereals, significant progress has been made over the past 2 decades towards inducing root modifications with structures resembling a legume nodule at least morphologically. When treated with low concentrations of various auxins, graminaceous crops such as wheat, rice, and maize etc. develop tumorous structures (paranodules) along primary and secondary roots. These structures or paranodules can be reproducibly induced on the roots of a range of plant species with 2,4-D (Ridge *et al.*, 1993). Glagoleva *et al.* (1997) claimed to have reported for the first time the formation of paranodules on the roots of rape (*Brassica napus*) due to 2,4-D and bacterial inoculation. The nodules formed on 2,4-D treated roots in the presence of bacteria were structurally different than those formed without inoculum. Increase in bacterial number and nitrogenase activity was also demonstrated. Indeed, 2,4-D has extensively been used to induce paranodulation in cereals and non-legumes in spite of the potential environmental objection to its use for field crops, even for localized applications on roots. It should be remembered, however, that 2,4-D is still extensively used as herbicide for broad-leaved plants and it is rapidly broken down in soil (Jackson, 1985). Fears of potential environmental damage may thus be exaggerated when balanced against the benefits of cereals like wheat and rice fixing their own  $\text{N}_2$ .

In some other studies, cell wall degrading enzymes, polyethylene glycol or genetically engineered rhizobia have been used to induce paranodules in rice seedlings and other non-legumes (Al-Mallah *et al.*, 1990). Cocking *et al.* (1993) found some naturally occurring strains of rhizobia that did not need enzymes for invading roots of these crops. These bacteria adopt crack entry mechanism instead of thread formation, which is characteristic of legumes.

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Contrary to bacterium-induced nodules, however, the ones formed as a result of treatment with enzymes had less resemblance with legume nodules and did not exhibit nitrogenase activity (Cocking *et al.*, 1990). Host specificity could also be removed by enzymatic treatment (Al-Mallah *et al.*, 1987), while adding microorganisms can modulate or interfere with the development of these outgrowths (Ridge *et al.*, 1992, 1993). These authors concluded that 2,4-D disturbs the orientation of cell division causing abnormal root growth. Different strains of *Rhizobium*, *Azospirillum*, *Agrobacterium* and *Escherichia coli* showed different effects on seedling health and on the growth and internal structure of these outgrowths. Generally, bacteria caused a less organized internal structure and an earlier senescence. Some bacteria also caused stunting and death of seedlings at concentrations of 2,4-D that would have no such effect when applied alone (Ridge *et al.*, 1992). Most of these studies on paranodulation are restricted to seedling stage and even that under hydroponic conditions. Recently, Azam and Lodhi (2001) have reported significant modifications in root system of wheat, rice and maize grown in soil. Some of the primary laterals were transformed into the so-called paranodules.

Histologically, auxin induced tumors or paranodules in cereals appear as cancerous grown out root meristems and thus comparable to stem nodules of *Sesbania rostrata*. Nie *et al.* (1992) have described in detail the structure of 2,4-D induced paranodules in wheat. They showed a central vascular tissue surrounded with several layers of cortical cells and connected to the root stele. These structures are also reported to develop a central vacuolar system similar to that in roots (Christiansen-Wengier, 1997). This is in contrast to the organization of vascular tissue in legume nodules in which the stele surrounds the infected cells. However, in tissue organization, paranodules of wheat resemble those of *Parasponia*, a non-legume (Trinick, 1988). Ridge *et al.* (1993) described in detail the formation of nodule like structures on roots of rice seedlings following exposure to 2,4-D. These structures had a smooth epidermis, distinct zoning of tissue, internal development of proto-vascular elements typical of a vascular bundle and a close resemblance to the structures induced in *Parasponia* by *Bradyrhizobium*. *Parasponia* is the only non-legume reported to be nodulated by *Rhizobia* (Trinick, 1973). The bacteria infect through the cracks created by lateral root emergence. Kennedy and Tchan (1992) suggested that crack entry or the entry through damaged root portions (e.g., due to 2,4-D) is the main route for endophytes. They found *Azospirillum* lacking exopolysaccharides (EPS) to be a better colonizer of wheat roots. According to them a process to facilitated evolution of para-nodulated wheat involving the step-wise genetic improvement of both partners may eventually lead to effective  $N_2$  fixation association. Katupitiya *et al.* (1995) also demonstrated that *A. brasilense* lacking surface polysaccharides were more able to establish colonization on wheat roots, especially when roots were treated with 2,4-D. Gough *et al.* (1997) reported reproducible crack entry of *Azorhizobium caulinodans* into roots of *Arabidopsis thaliana* where they occur in high frequency. Similar mode of entry into wheat roots treated with 2,4-D has been proposed for *Nostoc* (Gantar and Elhai, 1999). Interestingly, this mode of entry is shared by many legumes that grow under aquatic conditions (De Bruijn, 1995, Drayfus *et al.*, 1984). Attachment of rhizobia to rice roots (Terouchi and Syono, 1990), deformation of root hairs and formation of nodule like structures/hypertrophies (De Bruijn *et al.*, 1995, Rolfe and Bender, 1990) or thick short laterals (Cocking *et al.*, 1993) have been reported.

In rice, progress in achieving nodulation has been more significant than that in wheat. There is a fair degree of optimism that legume-rhizobium association could be extended to rice, as the later is able to enter into associations with mycorrhizal fungi (Secilia and Bagyaraj, 1992). Genetic links between the processes involved in nodulation and arbuscular mycorrhizae have been found in legumes (Gianinazzi-Pearson, 1996). Thus rice may possess part of the genetic machinery necessary for entering into mutually beneficial, endosymbiotic associations with other soil microorganisms. Rolfe and Bender (1990) used genetically modified

rhizobia to induce nodulation in rice. The structures thus formed were fairly similar to legume nodules with a good proportion of cells containing bacteroids. However, no significant nitrogenase activity was detectable. Primitive rice not so far bred for efficient fertilizer N use could be more susceptible to rhizobial interactions. Yanni *et al.* (1997) have reported such associations from Egypt in rice-legume rotations. Rhizobia from aquatic environments were more effective than terrestrial rhizobia in causing lateral root medications i.e., the formation of thick short lateral roots (TSLR), indicating that the primitive bacteria have a better nodule causing ability. Reddy *et al.* (1997) found that rice root exudates are unable to activate *nod* gene expression and exogenous application of chitolipooligosaccharide *nod* factors failed to elicit obvious morphological responses in rice roots. In spite of such difficulties, progress in developing some sort of  $N_2$  fixation system in rice has been made.

### Entry of bacteria into paranodules and their success as $N_2$ fixers:

Formation of nodular structures on roots of non-legumes and cereals has been reported since long but without any inference that these may contain bacteria. Significance of these structures induced by 2,4-D treatment in root-microbe interactions was recognized relatively recently by Nie and co-workers but without any acceptable evidence of  $N_2$  fixation (Nie *et al.*, 1992). The bacteria colonize intercellularly usually in the basal zone of the paranodules (Kennedy *et al.*, 1997) and there is no evidence of intracellular infection (Nie *et al.*, 1992) as is the case in legumes. In wheat, Kennedy *et al.* (1997) did not find intracellular infections with bacteroids, while significant infections were found only in 10-20% cases with most bacteria being located in pockets away from the central vascular system. Inoculation with rhizobia did not result in appreciable  $N_2$  fixation. In their review, Tchan and Kennedy (1989) also did not report any evidence of  $N_2$  fixation for these nodules in the presence of rhizobia. However, when *Azospirillum* instead of *Rhizobium* was used as the microbial partner, substantial amounts of  $N_2$  were fixed in wheat nodules obtained through 2,4-D treatment (Zeman *et al.*, 1992). Kennedy *et al.* (1991) found an effective colonization of paranodules by *Azospirillum* although a direct evidence of  $N_2$  fixation could not be obtained. Compared with this, studies by Christiansen-Weniger (1998) have reported  $NH_4$  excreting mutant of *A. brasilense* to survive inside these nodules in large numbers in maize and rice with a dense population, measurable quantities of N being fixed at the expense of C supplied by the host were also found. Colonization of wheat paranodules by several other bacteria has also been reported (Kennedy *et al.*, 1997). Using  $^{15}N$  methodology, Kennedy and Tchan (1992) obtained concrete evidence of  $N_2$  fixation in paranodulated wheat. Results similar to that of 2,4-D have been reported for naphthalene acetate (NAA) and colchicine (Kennedy *et al.*, 1991).

In spite of the success in inducing a kind of nodulation in wheat/rice (specially with 2,4-D) and some evidence of  $N_2$  fixation, the ability of the microsymbiont to benefit the plant has not yet been established. Mode of transfer of fixed N, longevity of nodules, and oxygen paradox etc., are some of the questions that need to be answered. Alternatively, the dogma of nodulation should be abandoned as suggested by Kennedy and Tchan (1992). Accidental or planned nodulation (tumor formation) can be caused in plants like rice and oilseed rape etc., but to make the system work towards  $N_2$  fixation has failed so far. This is because these plants do not have the necessary machinery to make the introduced bacterium work.

### Endophytic bacterial colonization of roots as an alternative to nodulation in cereals:

As mentioned earlier, partial success in causing root modifications somewhat similar to legume nodules has been achieved. However, this is not enough to extend legume-type nitrogen fixation to cereals and the scientists are quite sceptical about the absolute success in this direction. These views bear credence from the suggestion that genes controlling nitrogen-fixing symbioses of legumes with specialized bacteria known as rhizobia are presumably the products of many millions

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of years of evolution (Devine and Kuykendall, 1996). Kennedy and Tchan (1992) suggested that, "progress towards improved  $N_2$  fixation could be more likely if the "dogma" of legume nodulation is not used in future development". In their view, transfer of nodulation genes in cereals is a more ambitious and distant goal. Kennedy *et al.* (1997) are of the view that "at some point in time,  $N_2$  fixation may be transferred to crops such as corn and rice, but such an achievement must be seen as a far off goal". Similar views are shared by Burris (2000), who is of the opinion that "it does not appear that the solution to the problem of rice fixing its own nitrogen will come easily". All these views considered together with the account given above suggest that nodulation of a kind similar to that involving rhizobia and Frankia might be more difficult than previously thought. Thus a less developed nitrogen fixation association between bacteria and gramineae might be a preferable goal at this stage.

One of the potent approaches for increasing biological  $N_2$  fixation in cereals is to enhance associative and/or endophytic root colonization by desirable bacteria. Unfortunately, no definite evidence exists for the cause and effect of inoculation of associative organisms as variable results have generally been reported. The yield increases observed after bacterial inoculation are believed to be mostly due to the growth hormones rather than  $N_2$  fixation. There is inadequate N available for fixation, and the process is very inefficient and most of the N fixed will be available only slowly after the death and decay of the organisms (Azam *et al.*, 1989). An important consideration has also to be whether effective transfer of fixed N occurs to the plant; so far no evidence exists to this effect (Zimmer and Bothe, 1989).

When interactions between diazotrophic bacteria and graminaceous plants are studied within the context of trying to extend BNF to non-legumes the first step is to establish reproducible internal colonization of these plants at high frequency. The graminaceous plants known to have some, substantial or measurable  $N_2$  fixation, include rice, sugarcane, pearl millet, maize, sorghum, and kallar grass. This is significant, as most of these plant types love high moisture. Their success in meeting part of their N requirements through fixation may be the result of oxygen elimination under these conditions. Roots exposed to low oxygen partial pressure, as might occur during flooding and waterlogging of soil, become more leaky and loss of soluble C increases leading to higher bacterial population at the root surface (Tavaria and Zuberer, 1998). Hence, under high moisture conditions, the plant is not only providing enhanced amounts of carbon, but to a certain extent the oxygen paradox is also taken care of with the result that substantial amounts of  $N_2$  can be fixed by the associative or endophytic bacteria. An added benefit to the  $N_2$  fixers in terms of C supply is also the lack of competition by aerobic microorganisms under anaerobic conditions.

Hurek *et al.* (1994) found that apical region of the root behind the meristem was the most intensively colonized by bacteria. Indeed, the release of root border cells or root cap cells (BRD) is a common feature in most crops including wheat and serve as substrate/bate for bacteria/pathogens (Hawes *et al.*, 1991). Schlöter and Hartmann (1998) found effective colonization of root tip cells by different bacteria probably because of root cap cells or root border cells serving as the carbon source. Yanni *et al.* (1997) found good colonization of rice root interior by diverse types of rhizobia which could effectively nodulate clover (*T. alexandrinum*) and fix significant amounts of N. Certain rhizobial inoculants significantly improved different growth parameters of rice under both laboratory and field conditions. Since no attempt was made to determine contribution through  $N_2$  fixation, they hypothesized that the positive effects may be more through hormonal means. Gough *et al.* (1997) used *Azorhizobium* in such studies and found high intercellular endophytic colonization of both rice and wheat roots. Reasonable  $N_2$  fixation (using acetylene reduction assay, ARA) was detected and the inoculated plants showed better growth. Surface colonization by rhizobia (Chabot *et al.*, 1996) of cereal roots and their entry through cracks (Reddy *et al.*, 1997) has been reported. Christiansen-Weniger and Vanderleyden (1994) have described the colonization of maize roots by *Azospirillum*.

Treatment with 2,4-D is reported to correlate positively with increased internal bacterial colonization in wheat roots (Kennedy and Tchan 1992). The 2,4-D treated plants were shown to carry more associated azospirilla than untreated plants (Katupitiya *et al.*, 1995), a dense layer of bacteria was observed covering the surface of young parts of 2,4-D treated roots, nitrogenase activity was high especially when inoculum was also given. Christiansen-Weniger (1992) found considerable stimulation of  $C_2H_2$  reduction in 2,4-D treated wheat seedlings. They showed that auxin-affected portions of rice nursery are attractive infection sites for the bacteria, with enhanced  $N_2$  fixation. Using  $^{14}C$ , Elanchezhian and Panwar (1997) found higher translocation of photosynthates to the rhizosphere due to the 2,4-D treatments. Hence, one of the mechanisms whereby 2,4-D enhances bacterial colonization of roots may be through an increase in rhizodeposition by the plants probably due to cells becoming leaky. Kennedy and Islam (2001) proposed that 2,4-D has an effect akin to bacteria that produce cellulases and polygalacturonases in loosening as well as breaking the intercellular bonds. The cells thus released will add to the amount of rhizodeposition with a consequent increase in bacterial colonization of roots. Zeman *et al.* (1992) demonstrated that energy source required for  $N_2$  fixation by colonizers of wheat roots treated with 2,4-D was supplied by the plant. Nitrogen fixation associated with 2,4-D treated and inoculated seedlings was demonstrated using  $^{15}N$  as well as nitrogenase activity (Yu and Kennedy, 1995). Francisco and Akao (1993) observed 2,4-D to cause sloughing off of the epidermis as the cells proliferated from the actively dividing apical and adjacent lateral meristems. The sloughed off material can serve as a good C source for bacterial assimilation. Some of our unpublished work shows rapid proliferation of bacteria following transfer of wheat seedlings in the medium containing 2,4-D. Sabry *et al.* (1997) showed that wheat grown in pots and inoculated repeatedly with *A. caulinodans* showed colonization at the point of emergence of lateral roots and appeared to obtain significant amounts of nitrogen through fixation. By inducing many lateral root initials simultaneously, 2,4-D may increase the entry of microbes into the root interior and enhance  $N_2$  fixation.

An important factor encouraging bacterial proliferation and colonization will be when plant roots are exposed to some kind of stress. Indeed, 2,4-D treatment is reported to enhance stress tolerance of wheat (Gulnaz *et al.*, 1999). Smart *et al.* (1995) presented evidence to suggest that insufficient bacterial biomass exists on the root surfaces of non-stressed plants grown under well aerated conditions to quantitatively interfere with root nitrogen absorption measurements i.e., their contribution to N nutrition of plants may not be substantial. Thus any stress will make the plant exude more C into the rhizosphere with a consequent proliferation of rhizospheric bacteria. For example, high moisture, high salts, low moisture, lower than the required  $O_2$  level etc. will make the plant exude more. High moisture conditions in soil would not only damage the roots leading to more exudation, but to an enhanced bacterial colonization and an increase in the number of anaerobic bacteria. Under anaerobic conditions, certain types of bacteria would flourish in the absence of competition from the aerobes, which are more quick users of any available C source, e.g., that released from the roots.

The above account suggests substantial bacterial colonization of cereal roots, both surface and endophytic. The endophytes have the advantage of avoiding competition with rhizospheric population for rhizodeposits. Instead they are sitting at the site of exudates export and may thus benefit immediately from the C materials diverted to the roots. In order to obtain results of choice, the plants can be modified in a way that they support desirable microbial diversity. Indeed, a remarkable diversity of bacteria is found associated naturally with roots of field-rice (Yanni, 1991). Transgenic plants are reported to have a different effect on rhizospheric microbial diversity (Di-Giovanni *et al.*, 1999). In fact it is the plants control in the rhizosphere that some genotypes are resistant to soil-borne plant pathogens while others are not. Same will be true for  $N_2$  fixing plant types. Indeed, the so-called plant-microbe specificity will depend on specific compounds

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released into the rhizosphere and the resultant threshold level of the particular microbial population to achieve effective plant-microbe association. Co-inoculation of plant growth promoting rhizobacteria (PGPR) with effective rhizobia in chickpea resulted in significant increase in nodule weight and other plant characteristics.

In summary, endophytic root colonization could be achieved to the extent that measurable  $N_2$  fixation could take place. This mode of  $N_2$  fixation could be induced more conveniently in crops like rice, which grow under higher moisture conditions. Success in this direction has already been witnessed in sugarcane, which provides enough carbonaceous materials to support reasonable  $N_2$  fixation activity.

**Conclusions:** Significant progress has been made towards induction of rooting modification in rice and wheat that resemble root nodules of legumes. However, attempts have only marginally been successful to make these structures harbour sufficient bacterial population and provide an environment conducive for nitrogen fixation to occur. Further, the availability of fixed N to the macrosymbiont is still debatable. Thus induction of effective and functional nodulation in cereals appears to be a far off possibility. Alternatively, however, there is a fair degree of probability to induce endophytic root colonization. This process could be facilitated by using cell wall damaging chemicals, like 2,4-D. Optimism seems to prevail therefore that some sort of  $N_2$  fixing symbiosis could be achieved in cereals, particularly in rice. This will enable this important cereal fulfil part of its N requirements through bacterial fixation leading to savings in fertilizer inputs and consequently the environment.

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