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Stem nodulating *Sesbania rostrata* as a bioresource for rice production, induction of nodulation and endophytic N_2 fixation in rice.

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Abstract: The stem nodulating leguminous green manure *Sesbania rostrata* has great potential as an effective bioresource material for rice production due to higher biomass production and N_2 contribution. Attempts were made to extend the symbiotic N_2 fixation to non-legumes such as rice. The symbiont *Azorhizobium caulinodans* ORS 571 upon inoculation, stimulated the lateral root development as well as formation of nodular like structures in the roots of rice. Colonization of lateral root cracks by *A. caulinodans* ORS 571 was found to be stimulated by the flavonoid naringenin. Rice seedlings growing aseptically in the presence of naringenin were inoculated with *A..caulinodans* ORS 571 (pXLGD4) carrying the *lac Z* reporter gene. By microscopic analysis of sections of inoculated rice roots, it has been demonstrated that the xylem of rice roots can be colonized by *Azorhizobium caulinodans*. This could provide a suitable niche for endophytic nitrogen fixation by *A. caulinodans* in rice. (*Key Words*: *Azorhizobium caulinodans, Endophytic colonozation, Naringenin, Nodulation, Non-legumes, N_2 fixation, Sesbania rostrata, Xylem colonization*).

Nitrogen is the most critical nutrient for rice productivity. Rice takes 1 kg of nitrogen to produce 15-20 kg of grain. Low land rice in the tropics can use the nitrogen that is available either naturally through BNF or from mineralisation in soil to produce 2-3t of grain ha^{-1} (Watanabe and Roger, 1984; Ladha *et al.* 1993). However, additional nitrogen must be supplied for higher yield. Innovative research is needed to help keep rice supply ahead of demand while sustaining the world's natural resource base. To feed the ever increasing population, the world annual rice production must increase from the present 460 million to 760 million t by 2020 (IRRI, 1993). At present nitrogen use efficiency level, at least double the 10 million t of nitrogen fertilizer currently used each year for rice production will be required (IFA – IFDC – FAO, 1992; IRRI, 1993). Manufacturing the fertilizer for today's need requires 544 x 10^7 MJ of fossil fuel energy which is equivalent to 13 million t of oil, a non renewable source, the oxidized products of which pose hazards to human health and to the environment. With the gradual depletion of petroleum resources, increased production costs and low efficiency of chemical N fertilizer it is time to think of supplementary and or alternate sources of N for rice, besides exploring ways for efficient use of soil and fertilizer N, in order to meet increasing demands in coming years. It is in this context the BNF derived N assumes significance in the low land system that provide about 80% of the world's rice. Conventional BNF systems have only a limited capacity to render rice independent of external sources of N. Among the conventional BNF systems of rice, the free-living / associative diazotrophs have low to moderate potential to supply N because N_2 fixed outside the plant is subjected to loss. The amount of N_2 fixed by free- living and associative diazotrophs in rice field are low and inefficiently utilized as compared with those of legumes under suitable conditions. If a BNF system could be assembled in non-legumes such rice, wheat, maize and *Sorghum* as in sugarcane, we could enhance the N supply potential as fixed N would be available directly to the plant with little or no loss. Thus it has become imperative to consider unconventional BNF systems to supply rice with N.

Association of diazotroph with rice can perhaps be improved if a niche for harbouring the bacteria can be created in the plant. Many plant species including some monocots, develop paranodules to 2,4-D (Kennedy and Tchan, 1992). Shizhen and Donguwai (1994) and Zhiguo *et al* (1994) reported that colonization and N_2 fixation by *A. caulinodans* ORS 571 in the 2,4-D induced paranodules was less senstive to oxygen than in non paranodulating roots. Yu and Kennedy (1995) reported colonization and N_2 fixation by *A. caulinodans* ORS 571 in wheat. However it is yet to be proved whether such an association will have agronomic applications since it is not known whether the introduced diazotrophs can selectively colonize chemically induced paranodules in natural condition (Christiansen – Weniger, 1996).

Preliminary studies were made to find out the feasibility of extending the symbiotic N_2 fixation to cereals such as rice, *Sorghum*, baby corn and maize. These studies revealed the formation of nodule like structures in roots of rice, maize and *Sorghum* (Chandrasekar and Kannaiyan, 1995), when inoculated with *A. caulinodans* and growth regulator 2,4-D at 0.5, 1.5 and 3.0 ppm. There was no nodular like structure formation when either *A. caulinodans* or 2,4-D alone was applied. Further research revealed the stimulation of lateral roots formation in rice, maize and cumbu upon inoculation with *A. caulinodans* in the presence

of growth regulators 2,4-D, NAA and Kinetin (Amutha and Kannaiyan, 1998).

Sesbania rostrata

The stem nodulating tropical legume, *Sesbania rostrata*, is a promising green manure species for low input rice farming systems in lowland rice. Many investigators have reported that *Sesbania rostrata* adopt well in flooded rice soils (Dreyfus and Dommergues, 1981; Duhoux and Dreyfus, 1982 and Kannaiyan and Kalidurai, 1989). Dreyfus *et al.* (1984) reported that the stem nodulating *Rhizobium* in *S. rostrata* is *Azorhizobium caulinodans*. Becker *et al.* (1986) reported that stem nodulating ability of *S. rostrata* provides an opportunity to fix nitrogen under water logging and high soil nitrogen conditions. *S. rostrata* could add substantially higher amounts of nitrogen than is required by rice crop (Ladha *et al.* 1988). Besides producing an economic seed yield, it is known to contribute significant amounts of soil nitrogen in rice fields (Buresh and De Datta, 1991). Balasubramani *et al.* (1992a) have reported that nitrogenase activity was significantly higher in stem nodules than in the root nodules of *Sesbania rostrata*.

Ladha *et al.* (1989) reported the ability of *S. rostrata* to produce nodules on stem, in addition to the root nodules, is an advantage. In stem nodulating plants like *S. rostrata*, nodules are produced on the above ground part of the plant where competition with other rhizobia was negligible. Therefore, stem nodulation in *S. rostrata* could be effectively enhanced by suitable *Rhizobium* strain (Fleischman *et al.* 1991). The common characteristic of all stem nodulated legumes is the presence of pre-determined nodulation sites on the stem. Nitrogen fixing stem nodules always occur at the emergence of lateral root primordia and the ruptured tissues in this region constitute the site of entry for the *Rhizobium* (Arora, 1954; Duhoux and Dreyfus, 1982; Kannaiyan and Kalidurai, 1989 and Chitra and Kannaiyan, 1994).

The rhizobial cells reach the primordia of *S. rostrata* and penetrate the intracellular spaces and multiplies concurrently with the meristematic activity of the cells (Dreyfus *et al.* 1985). Tsien *et al.* (1983) observed the formation of branches of intracellular infection thread during the development of nodules of *S. rostrata*. Ladha *et al.* (1989) showed that azorhizobia released from stem nodules can survive and grow in flooded conditions and colonize the rhizosphere and histosphere of rice. Adebayo *et al.* (1989) reported the presence of large population of *Azorhizobium caulinodans* on leaves and flowers of *Sesbania rostrata*.

Ladha *et al.* (1988) found the presence of *Azorhizobium* in the spermosphere of *Sesbania rostrata* and the same could not be eliminated by treatments normally used for seed sterilization and scarification. *Azorhizobia* in seeds may come from senescing stem nodules during flowering and seed formation. As nodule formation occur on flowering branches, flower may be contaminated with rhizobia; these may enter the seed through the hilum scar, pilar groove and micropyle. It can be hypothesized that the mode of initial entry of azorhizobia into the soil is through the seeds (Ladha *et al.* 1990). The occurrence of *A. caulinodans* in seed and other plant parts of *S. rostrata* was also reported by Balasubramani and Kannaiyan (1991a). Becker *et al.* (1990) reported that rain water can wash the epiphytically growing rhizobia down the stem and thereby infect nodulating sites.

Dreyfus and Dommergues (1981) considered rain as a possible means to inoculate primordia on the stem of *S. rostrata* under non-flooded conditions. Ladha *et al.* (1989) have reported that the mode of initial entry of azorhizobia into the soil is through rain splash and insect activity. The nodules formed on the stem of *S. rostrata* may release azorhizobia on senescence and survive epiphytically (Adebayo *et al.* 1989). Robertson and Alexander (1994) suggested that rain and wind blown soils are the means of transmitting the bacterium, *A. caulinodans*, thus favouring stem nodulation.

In TNAU, much work has been made on the isolation and characterization of efficient isolates of *Azorhizobium caulinodans* from both *S. rostrata* and rice soil previously cropped with *S. rostrata*. In earlier times, stem nodules in *S. rostrata* has attracted much attention in terms of biomass production and nitrogen contribution to wet land rice (Kannaiyan *et al.* 1988; Kalaidurai and Kannaiyan, 1989; Kalidurai and Kannaiyan, 1991a; Balasubramani and Kannaiyan, 1991a, 1992; Sundaravarathan and Kannaiyan 1998; Kannaiyan *et al.* 1988). Kalidurai and Kannaiyan (1991b) have reported that both stem and root isolates of *A. caulinodans* could tolerate combined nitrogen at 2.5 ppm and fix N_2. *S. rostrata* callus inoculated with *A. caulinodans* showed nitrogenase activity under *in vitro* conditions (Balasubramani, 1990; Chitra, 1992; Amutha, 1997). The epiphytic occurrence of *A. caulinodans* in *S. rostrata* plant parts including leaves, stem, flowers, pistil was reported (Balasubramani and Kannaiyan, 1991 a, b; Balasubramani, *et al.* 1992 b, c). Interestingly the bacterium *A. caulinodans* was isolated from the various parts of ants, mealy bug and beetles (Kannaiyan, 1997).

The movement of *A. caulinodans* within *S. rostrata* was studied and established by isolation from different plant parts of inoculated *S. rostrata* (Chitra and Kannaiyan, 1994). They also reported the presence of *A.caulinodans* both in the rice rhizoplane region and in rice soil.

Azorhizobium caulinodans

Nitrogen – fixing nodules are usually found on the roots of the host leguminous plants. Only some legume species belonging to the genera *Neptunia, Aeschynomene* and *Sesbania* bear nodules on both roots and stem. Dreyfus *et al.* (1988) found that the tropical legume *Sesbania rostrata* was actually associated with *Azorhizobium caulinodans.*

The cells of *A. caulinodans* are gram – ve, small rods (0.5 – 0.6 µm by 0.5 – 2.5 µm) and motile. The cells have peritrichous flagella on solid medium and one lateral flagellum in liquid medium (Dreyfus *et al.* 1988) On agar media, the colonies appear circular, viscid and creamy colour and fix N_2 in microaerophilic condition. Bacteria growing within the para-nodule displayed variable morphology, differing in size and cell contents. Electron transparent regions within some bacterial cells of these sections may have accumulations of Poly-β-hydroxybutyrate. Considerable amounts of indeterminate material were found together with these bacteria. This was surprising, since the bacteria were extracellular in the middle lamellar space or in rare cases, present in dead cells. *Azorhizobium caulinodans* ORS 571 induces the formation of root and stem nodules on the tropical legume *Sesbania rostrata. Azorhizobium* can use nitrogen while free living and organic acids such as lactate and succinate are the favorite carbon substrates for both NH_4^+ and N_2 dependent growth.

This bacterium contains essential nodulation genes as that of *nod ABC* of rhizobia (Goethals *et al.* 1989). Hence it is possible to elucidate the *nod* genes in the biosynthesis of the Nod factor induced by host plant exudate. In view of reports of associative N_2 fixation by *Azorhizobium* in rice fields, it will be interesting to study this behaviour and identify functions that contribute to the interactions between *Azorhizobium* and rice plants. The induction of paranodules and the invasion of rice by rhizobia ,either spontaneously or after the treatment of the roots with cell wall degrading enzymes have been reported (Cocking *et al.*1990).The nodule like structures induced by 2,4-D can be inhabited by diazotrophs (Nic,1983). It is possible that 2,4 -D has at least two effects:

1. An auxin-like activity, controlling development of nodular structure;
2. An effect enabling increased frequency of infection of plant root cells and colonization of nodular structures with certain bacteria.

The choice of the bacterial strain *A. caulinodans* ORS 571 was based on three characteristics (de Bruijn, *et al.* 1995). Firstly as it is unlikely that there will be bacteroid differentiation in non-legume plants, ORS 571 has the unusual ability among the rhizobia to fix nitrogen in free-living conditions without differentiation into bacteroids. Secondly, as it is also unlikely that non-legumes will be able to reproduce the sophisticated oxygen barriers which exist in legume nodules to protect the nitrogenase enzyme complex from inhibitory oxygen levels, ORS 571 can tolerate up to 12 µM dissolved oxygen while fixing nitrogen in culture. Thirdly ORS 571 forms nodules on its host plant following crack entry infection i.e. inter-cellularly between adjacent cells. It has been proved that the *Azorhizobium caulinodans* strain ORS 571 is able to enter the hosts (rice, wheat, *Sorghum*, oil seed rape and *S. rostrata*) through crack entry at the point of emergence of lateral roots, their colonization and invasion was significantly stimulated by the flavonoid naringenin. Though in rice *Azorhizobium caulinodans* was found to stimulate the root – hairs formation, no root – hair curling was observed. However abnormal lateral root development was observed within 2 weeks after inoculation. More than 80% of the rice roots exhibited thick short lateral root (TSLR).

Flavonoid as signal molecule for endophytic colonization by A.caulinodans

Flavonoids are a class of phenolics secreted by plant roots as part of the regulatory system for colonization of *A. caulinodans*. Four families of flavonoids such as flavones, flavonols, flavonones and iso-flavonones have been shown to have nod-gene inducing activity. Naringenin - a specific flavonoid is known to stimulate the colonization of *A. caulinodans* in rice roots. It is a signal molecule for attracting the population of *A. caulinodans*. But rice root did not produce the flavonoid naringenin. The flavonoid naringenin is considered as a potential signal molecule to activate the colonization of *A. caulinodans* in rice roots and, therefore, a specific gene could be introduced into rice plants for synthesis of naringenin. Flavonoids also enhance the growth rate of certain rhizobia and phenolic compounds in general could induce expression of a variety of genes in plant associated bacteria. The flavonoids naringenin and diadzein, at low concentration were found to significantly stimulate the frequency of LRC and inter-cellular colonization by *A. caulinodans* in non-legumes by acting as chemical

stimulants (Gough *et al.* 1996). Flavonoids have been shown to accumulate in many plants upon infection. However, there are few reports showing such accumulation in cereal crops. Naringenin was detected in rice leaves exposed to UV irradiation. Kumar *et al.*(1996) while studying the effect of flavonoid naringenin on the endophytic colonization by *A. caulinodans* in rice, reported favourable stimulation of lateral root formation by naringenin in ADT-36 rice, They also reported that the main mode of entry of *A. caulinodans* in rice roots is by crack entry through the emerging lateral roots. The effect of naringenin,enzyme mixture and *A.caulinodans* on induction of para nodules in rice and maize was reported (Senthil Kumar,2000).It was found that addition of naringenin enhanced the short thickened lateral root formation. The seedlings treated with naringenin ,enzyme mixture and *A. caulinodans* recorded maximum number of lateral rootlets, rhizoplane population, nodule like structures and nitrogenase activity. Lateral root development of rice and colonization of lateral root cracks by bacteria were shown to be stimulated by the flavonoid naringenin. The effect of flavonoid on root morphology of different rice varieties was studied and found that flavonoid naringenin at 5 x 10^{-5} M concentration significantly stimulated the lateral root formation in rice varieties (Gopalaswamy *et al.* 2000).

Endophytic colonization by A.caulinodans

"Endophytic nitrogen fixation" is a process where the nitrogen fixing bacteria enter the plant roots through cracks or fissures in the lateral roots, colonize the inter-cellular spaces (cortex, within few cells of epidermis, inner cortex to epidermis, vascular tissues and xylem), without differentiating into bacteroids fix atmospheric nitrogen micro-aerobically, with the help of the enzyme "nitrogenase" (Kennedy *et al.* 1997). In the earlier days, it was thought that only by inducing nodular structures to provide a place for the organism specifically, BNF can be extended to cereals (Arora, 1954). But now recent researches have vividly brought down the difficulties and increased the scope for N_2-fixation in cereals by means of endophytic nitrogen fixation (Stoltzfus *et al.* 1997; Kannaiyan, 2000).

The extension of biological nitrogen fixation to non-legume crops such as rice, maize *etc.* would be of enormous economical and environmental impact. The discovery that bacteria of the genera *Rhizobium* and *Bradyrhizobium* can form effective nitrogen fixing nodules on the non-legume *Parasponia* (Davey *et al.* 1997) provided added impetus to this approach. Many of these nodular structures have to be artificially induced by adding 2,4-D (Kennedy *et al.* 1990; Tchan *et al,* 1991) or cell wall degrading enzymes (Al-Mallah *et al,* 1989; Cocking *et al,* 1990; Tchan *et al.* 1991). Cocking *et al.* (1993) reported that *Rhizobium* of *Parasponia* and *Aeschynomene* produce nodule like structure on the emerging lateral roots of rice and maize without any enzyme treatment. Kennedy and Tchan (1992) studied various approaches for the induction of nodule like structures on the roots of rice and other cereals.

Zeman *et al.* (1992) reported that the exogenous application of the synthetic auxin 2,4-D to rice induces modified root outgrowths (MROs) which result from the induction of meristem. Reports have been made that certain rhizobia can enter rice at low frequency (Cocking *et al,* 1994). Christiansen-Weniger (1996) reported nodule like structure formation in rice roots treated with 2,4-D and inoculated with *A.caulinodans*. It is suggested to have a thorough knowledge about the plant genes involved in nodulation and symbiotic nitrogen fixation and the function of their gene products before the development of true nodulation of cereals (de Bruijn *et al.* 1995; Kennedy *et al.* 1997; Webster *et al.* 1997). Now, opinion has consequently changed and the emphasis is to try to establish stable endophytic associations between diazotrophic bacteria and non-legume crops.

Two major approaches are currently being undertaken to try to extend biological nitrogen fixation to non-legume crops. The first is to look for natural endophytic diazotrophs of non-legume crops and to assess such endophytic nitrogen fixation. This is being exploited with sugarcane (Boddey *et al.* 1995) and other crops such as rice and wheat (Sabry *et al,* 1997). The second approach is to determine whether the rhizobia can internally colonize and ultimately fix nitrogen in non-legumes. The first step is to study the possibility of inter-cellular colonization of diazotrophs with non-legumes including entry, spread and the mechanism involved. A sensitive and quantifiable system is needed for this. Microscopic observation of colonization is greatly facilitated by using a genetically modified bacterial strain tagged with a reporter gene (Webster *et al.* 1997).The efficacy of endophytic colonization by diazotrophs *Azospirillum* and *Azorhizobium* tagged with *lac* Z marker genes in rice was compared. It was observed that the colonization by diazotrophs was mainly through crack entry. In the case of *Azospirillum* the bacteria entered the lateral cracks and their invasion was not so deep and very much limited to endodermis and cortex, whereas, *Azorhizobium* exhibited deep colonization. They first got entry through lateral cracks and traverse intercellularly and colonized xylem of rice var. ADT 36 (Gopalaswamy *et al.* 2000).

Prerequisites for an endophytic relationship

There are several prerequisites for the establishment of an endophytic situation: (Quispel, 1991)

i) Hostile reactions from the host, like hypersensitive reactions and production of phytoalexins have to be prevented.

ii) The bacteria must find an inter or intracellular niche, where sufficient reducing conditions are provided and the pO_2 is regulated by diffusion barriers and/or leghaemoglobins.

iii) Membrane systems around the endosymbiont of microbial and host origin must be adapted in such a way that a bi-directional transport of substances is possible

Recently Chaintreuil *et al.* (2000) have investigated the presence of endophytic rhizobia within the roots of the wetland wild rice *Oryza breviligulata*, which is the ancestor of the African cultivated rice *Oryza glaberrima*. This primitive rice species grows in the same wetland sites as *Aeshynomene sensitiva*, an aquatic stem – nodulated legume associated with photosynthetic strains of *Bradyrhizobium*. Twenty endophytic and aquatic isolates were obtained at three different sites in West Africa (Senegal and Guinea) from nodal roots of *O. breviligulata* and surrounding water by using *A. sensitiva* as a trap legume. Most endophytic and aquatic isolates were photosynthetic and belonged to the same phylogenetic *Bradyhizobium, Blastobacter* subgroup as the typical photosynthetic *Bradyrhizobium* strains previously isolated from *Aeschynomene* stem nodules. Nitrogen fixing activity, measured by acetylene reduction, was detected in the rice plants inoculated with endophytic isolates. A 20% increase in the shoot growth and grain yield of *O. breviligulata* grown in a green house was also observed upon inoculation with one endophytic strain and one *Aeschynomene* photosynthetic strain. The photosynthetic *Bradyrhizobium* sp. strain ORS 278 extensively colonized the roots surface, followed by intercellular and rarely intracellular, bacterial invasion of the rice root, which was determined with a *lac*-Z-tagged mutant of ORS-278. The discovery that photosynthetic *Bradyrhizobium* strains, which are usually known to induce nitrogen – fixing nodules on stem of the legume *Aeschynomene*, are also natural true endophytes of the primitive rice *O. brevigulata*, could significantly enhance cultivated rice production.

Para nodulation by A.caulinodans

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. The results of experiments carried out in China on the induction of "nodule – like structures" on rice roots by rhizobia were highlighted. They concluded that the formation of hypertrophies on rice roots infected and colonized with microbes had been confirmed, but that little evidence supported their designation as "nodules" or even nodule – like. The frequency of formation of the roots 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.

During the past four years, the status of biological nitrogen fixation in non-leguminous field crops were reviewed (Kennedy, 1994 and 1997; Katupitiya *et al.* 1995a, 1995b; Yu and Kennedy,1995 and Kennedy *et al.* 1997). The approach is based on the initial observation of 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 plants species.

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 colonization and nitrogen fixation 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 indole acetic 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).

Para – nodular bacteria -Mode of entry and colonization

Para-nodules are nodular structures produced by modification from the lateral roots of many plant species due to the stimulating effect of auxins such as 2,4-D (Tchan and Kennedy, 1989). These structures inhabited by diazotrophs may be quite dissimilar to legume nodules (Kennedy and Tchan, 1992).

The invasion of root cells by endophytic bacteria and their colonization have attracted the interest of many workers. The bacteria are attracted by certain root exudates. In cereals like rice and maize, since the exopolysaccharide production and the production of flavonoids like naringenin, luteoline and daidzein are low, by supplying them artificially at very low concentrations invasion

can be enhanced. (Kennedy *et al.* 1997; Sabry *et al.* 1997).The bacteria searches for cracks or fissures through which it can enter the roots. The bacteria enters through cracks in the lateral roots. It moves through the intercellular spaces and finds a site of low redox potential, possibly better suited for N_2 -fixation. A large number of bacteria were also found associated with the para-nodules (Kennedy *et al.* 1997).

Structure of para-nodules

Para-nodular structures were formed when seedlings were treated with 2,4-D. The para-nodules were spheroidal in shape, and less than a millimeter in diameter. The frequency of para nodulation on the young roots was 1-4 nodules per cm of root length. Sections of para-nodules showed the presence of central vascular tissue, connected to the root style. Several layers of cortical cells, usually without recognizable cytoplasmic contents, surrounded this rudimentary stele. This is in contrast to the organization of vascular tissue in legume nodules, in which stele surrounds the infected cells. In early stages, root-cap like structures were found on the para-nodules. In later stages, outer cells in the region showed swelling and sloughed off frequently. Occasionally bacteria were observed inside the outer 'empty' cortical cells but never in large numbers. In contrast, extensive bacterial infection was confined to the inter-cellular spaces. Serial sectioning of para-nodules showed that the bacteria and associated material were present in large, irregular channels open to the exterior. Examination of roots by phase-contract and light microscopy indicated an extensive colonization of the root surface and root hair by the endophytic bacteria.Electron microscopical observation of the paranodules revealed the presence of large numbers of bacteria in inter-cellular spaces. These irregular spaces or channels were developed by splitting of the middle lamella, during bacterial colonization. These channels were surrounded by shrunken and almost empty cortical cells. Only cytoplasmic debris was observed in these empty cells.

Effect of growth regulators and cell wall degrading enzymes on induction of para nodules in rice

The inoculation of the bacterium *A. caulinodans* with growth regulators produces lateral rootlets formation in rice. During the development of lateral root emergence a minute way is formed on both sides of the lateral root by which the bacterium *A. caulinodans* could colonize well and invade into the roots through crack entry and gradually reach to the inner region of the rice root tissues. Seven days old rice seedlings of ADT 36, when inoculated with the symbiotic nitrogen fixing bacterium , *A. caulinodans* along with the growth regulators such as 2,4-D, NAA, kinetin could induce the formation of nodular structures. Buvana and Kannaiyan (1998) studied the effect of growth regulator NAA and cell wall degrading enzymes (cellulase and pectinase) on induction of para nodules in rice by *A. caulinodans*. Inoculation of *A. caulinodans* with NAA and cell wall degrading enzyme mixture (cellulase + pectinase) decreased the root growth of seedlings compared to inoculation of *A.caulinodans* alone. Inoculation of *A. caulinodans* and cell wall degrading enzyme mixture developed lateral rootlets which were short and thickened. The number of nodule like structures and total nitrogen content was maximum with inoculation of *A. caulinodans* strains in combination with cell wall degrading enzyme mixture and NAA (Table 1 and 2). Inoculation of *A. caulinodans* was found to increase the root and shoot growth, total biomass and lateral rootlets of rice seedlings over uninoculated control. The growth regulator kinetin was used at 3.0, 5.0 and 7.0ppm concentration and the levels above 3.0 ppm was found to be inhibitory to the seedlings by recording decreased root and shoot growth, total biomass and lateral rootlets. The inoculation of *A. caulinodans* could nullify the adverse effect of kinetin to some extent compared to the incorporation of growth regulator treatment alone. Increased concentrations of kinetin with inoculation of *A. caulinodans* increased the number of nodule like structures in the roots of young rice seedlings. The number of nodule like structure were relatively more in rice variety TKM 9 compared to the rice variety ADT 36 (Table 3). It is interesting to note that the increased concentrations of kinetin with inoculation of *A. caulinodans* increased the number of nodule like structures in rice seedlings. However, there is no nodule like structure formation in rice roots when *A. caulinodans* and growth regulator kinetin were treated in the seedlings separately (Amutha and Kannaiyan, 2000).

Xylem colonization by A. caulinodans in *S. rostrata*

Rhizobia are bacteria that invade legumes after activating cell division in the plant root cortex, initiating a new organ, the nitrogen – fixing nodule (Long, 1996). Since the nodule is generally considered the only endophytic destination of invading rhizobia, most previous studies have focused on the rhizobial invasion pathway into and within the cortex (Kijne, 1992). Many non-rhizobial endophytic bacteria colonize the vascular system of plants, sometimes, but not always leading to plant disease (Bell *et al.* 1995). For example, the xylem of healthy alfalfa, a legume, is colonized by non – rhizobial endophytes (Gagne *et al.* 1987). Agrobacteria, plant pathogens closely related taxonomically to rhizobia, invade

the xylem of several species including, interestingly the topical legume *Sesbania rostrata* (Vlachova *et al.* 1987). Inoculation of *S. rostrata* with *Azorhizobium caulinodans* ORS-571 (Dreyfus *et al.* 1988) results in production of nodules on roots (Ndoye *et al.* 1994) and stems (Tsien *et al.* 1983). Interestingly, ORS 571 is able to establish itself endophytically in the roots of rice (Christiansen-Weniger 1996) and wheat (Sabry *et el.* 1997). The presence of azorhizobia in the xylem was confirmed by light and electron microscopy. The recent availability of various genetic tools and reporter genes based on fusions with *lac* Z and *gus* A is having a major impact on studying the xylem colonization (Vande Broek *et al.* 1993; Arsene *et al.* 1994 and Gough *et al.* 1996). Bacteria re-isolated from inoculated plants and plated onto selective media containing X-Gal, produced blue staining colonies, confirming that some azorhizobia still retained pXLGD4. Re-isolated bacteria also exhibited typical azorhizobial colony morphology on semi-solidified TY medium and failed to take up Congo Red dye (a diagnostic test for rhizobia; Somasegaran and Hoben, 1994). Recently it was found that ORS-571 colonizes xylem elements, in addition to inducing and invading nodules in the root cortex of *S. rostrata*. However xylem colonization is not regulated in the same way as nodulation. Thus, for the first time, a species of legume nodule bacteria has been found to colonize, reproducibly, regions of the host plant other than nodules. This novel endophytic interaction will be of interest to phytopathologists, to researchers investigating legume-rhizobia interactions, and to workers attempting to extend endophytic rhizobial nitrogen fixation to non-legumes. (O'Callaghan, *et al.* 1997).

Xylem colonization by A.caulinodans in rice

The diazotroph *Azorhizobium caulinodans* ORS-571 colonizes xylem of roots of the legume *Sesbania rostrata*(O' Callaghan *et al.* 1997) in addition to colonizing nodules in that legume led us to investigate whether this rhizobial strain, might nevertheless be able to colonize the xylem of rice roots. Colonization of the xylem of rice by *A. caulinodans* ORS-571 is of interest because it might provide a suitable nutritional and environmental niche for nitrogen fixation, comparable to the situation in sugar cane in which endophytic diazotrophs internally colonize the plant, including the xylem, and are probably major contributors to the observed high levels of nitrogen fixation (Boddey, 1995).

A large number of plant species exhibit xylem colonization by bacteria without disease symptoms and xylem colonization is increasingly being seen as a common aspect of plant – microbe interactions (Hallmann *et al.* 1997). Moreover, it is becoming increasingly realized that the xylem may be more robust structurally and physiologically than previously envisaged and that it should no longer be regarded as a vulnerable pipeline on the edge of disaster (Canny, 1998). The non – rhizobial diazotrophic *Acetobacter diazotrophicus* has been shown to penetrate sugar cane roots intercellularly at the root tip and at cracks in lateral roots junctions and to colonize xylem vessels following the inoculation of aseptically grown plants (James *et al.* 1994). In the presence of the flavanoid naringenin, *A. caulinodans* ORS 571 entered the rice through lateral root cracks and present in large numbers in cortex (Webster *et al.* 1997). Reddy *et al.* (1997) have also observed that azorhizobia invade intercellularly the outer cracks. Little is known, however, as to how exactly bacteria reach and invade the xylem. It has been suggested that xylem elements are possible sites of nitrogen fixation by diazotrophs, since the xylem elements could provide the low pO_2 and a site for exchange of metabolites necessary for nitrogen fixation (James *et al* 1994; Rolfe *et al.* 1998). Recently Gopalaswamy *et al.* (2000) performed microscopic analyses of sections of the junction regions of lateral and primary roots of rice inoculated in the presence of naringenin with ORS 571 (pXLGD4) carrying the *lac* Z reporter gene and demonstrated for the first time the xylem colonization of rice roots by *A. caulinodans*. In fact, the number of plant species found to exhibit xylem colonization by bacteria without plant symptoms has increased dramatically in recent years, suggesting the xylem colonization is a common aspect of plant–microbe interactions (Kloepper *et al* 1992). Since *A. caulinodans* ORS-571 is able to fix nitrogen in the free-living state without differentiation into bacteroids up to 3% oxygen (Kitts and Ludwing 1994), this first report of the colonization of xylem elements of rice by azorhizobia may be of significance in this respect. The pO_2 in rice roots is likely to be influenced by waterlogging of the rice plant and more extensive invasion will probably be required to have an impact on the nitrogen balance.

Ecological and economic aspects

Here it has to be considered whether under field conditions, these non-legumes show the properties that would be expected on the basis of green house experiments. As it happens in the case of legumes, it is now being realized that these endophytes also aid in improving soil properties (Dobereiner *et al.* 1995). The crop residues and stubbles is a major route for the transfer of fixed nitrogen to the soil. The decomposition of dead roots and nodules, and a possible excretion of nitrogen have also to be considered (Rinaudo *et al.* 1983). It is thought that these organisms

Table 1. Effect of NAA and inoculation of *A.caulinodans* strains with enzyme mixture (cellulase + pectinase) on the number of lateral rootlets (LR) and the number of nodule like structures (N) of rice (ADT 36)

Treatments	RB-SK-1		RB-SK-2		RB-SK-3		RB-SK-4		RB-SK-5		RB-SK-6		RB-SK-7		RB-SK-8		RB-SK-9		RB-SK-10	
	LR	N	LR	N	LR	N	LR	N	LR	N	LR	N	LR	N	LR	N	LR	N	LR	N
Uninoculated	64.67	—	65.67	—	63.00	—	67.67	—	65.33	—	64.67	—	65.00	—	66.00	—	62.33	—	65.33	—
A.caulinodans (*A.c.*) alone	86.00	—	85.33	—	83.33	—	87.00	—	85.00	—	87.33	—	84.33	—	86.00	—	81.67	—	84.33	—
NAA(3.0ppm)	55.67	—	53.00	—	57.33	—	51.33	—	52.00	—	54.00	—	54.67	—	56.67	—	57.33	—	57.33	—
NAA+*A.c.*	81.00	32.00	82.00	34.67	84.33	29.33	82.67	32.33	82.33	34.00	84.00	31.67	84.33	33.00	85.00	—	80.67	34.33	82.00	34.23
NAA+*A.c.*+Cellulase	57.33	24.33	58.67	23.00	59.33	21.00	57.00	25.00	56.67	25.67	60.67	23.33	57.67	27.33	60.00	—	60.33	24.67	61.67	23.33
NAA+*A.c.*+Cellulase+Pectinase	77.67	35.33	78.67	32.00	76.00	33.00	76.67	34.33	75.00	36.00	79.67	33.33	77.00	35.33	80.67	—	74.67	36.00	74.00	35.33
NAA+*A.c.*+Pectinase	58.00	23.33	56.67	18.33	57.67	23.00	56.67	21.33	55.33	21.33	56.33	22.33	56.66	24.67	57.67	—	57.00	24.00	57.33	22.00
A.c.+Cellulase	65.67	—	68.33	—	68.33	—	70.00	—	64.00	—	68.33	—	70.67	—	70.67	—	66.00	—	65.67	—
A.c.+Pectinase	65.33	—	64.67	—	68.67	—	65.00	—	63.33	—	68.33	—	66.33	—	65.67	—	65.00	—	64.00	—

	LR		N	
	SEd	CD	SEd	CD
Treatment	0.34	0.66	0.61	1.25
Culture	0.35	0.70	0.99	1.98
Treatment x Culture	1.06	2.09	1.99	3.95

Table 2. Effect of NAA and inoculation of *A.caulinodans* strains with enzyme mixture (cellulase + pectinase) on the total nitrogen content of rice (ADT 36)

Treatments	Total nitrogen content (%)									
	RB-SK-1	RB-SK-2	RB-SK-3	RB-SK-4	RB-SK-5	RB-SK-6	RB-SK-7	RB-SK-8	RB-SK-9	RB-SK-10
Uninoculated	0.217	0.220	0.213	0.222	0.223	0.226	0.217	0.213	0.224	0.223
A. caulinodans (*A.c.*) alone	0.268	0.318	0.305	0.318	0.317	0.318	0.295	0.296	0.312	0.310
NAA (3.0 ppm)	0.188	0.181	0.175	0.176	0.182	0.180	0.178	0.182	0.180	0.180
NAA + *A.c.*	0.364	0.373	0.375	0.374	0.378	0.365	0.365	0.344	0.377	0.363
NAA+*A.c.*+Cellulase	0.330	0.349	0.356	0.343	0.359	0.348	0.335	0.325	0.353	0.324
NAA +*A.c.*+Cellulase+Pectinase	0.447	0.456	0.454	0.457	0.459	0.451	0.446	0.349	0.456	0.441
NAA+*A.c.*+Pectinase	0.313	0.335	0.325	0.336	0.335	0.327	0.320	0.325	0.339	0.318
A.c. + Cellulase	0.224	0.242	0.242	0.248	0.239	0.233	0.226	0.231	0.236	0.214
A.c.+ Pectinase	0.224	0.239	0.235	0.237	0.232	0.226	0.229	0.228	0.231	0.221

	SEd	CD
Treatment	0.002	0.004
Culture	0.002	0.004
Treatment x Culture	0.006	0.012

Table 3. Effect of kinetin and inoculation of *A. caulinodans* ZB-SK-5 on the number of nodule like structure of rice

Treatment	Para nodules (Numbers)			
	ADT 36	Purple Puttu	ASD 16	TKM 9
Uninoculated	-	-	-	-
A. caulinodans (A. c.) alone	-	-	-	-
Kinetin (3.0 ppm)	-	-	-	-
Kinetin (3.0 ppm) +*A. c*	20.67	22.67	20.33	25.33
Kinetin (5.0 ppm)	-	-	-	-
Kinetin (5.0 ppm) +*A. c*	31.67	31.67	27.00	35.30
Kinetin (7.0 ppm)	-	-	-	-
Kinetin (7.0 ppm) +*A. c*	46.00	46.67	48.00	50.30
CD (P= 0.05)	3.71	4.82	4.91	7.01

have an ability to act as pioneers in nitrogen poor soils and subsequently raise the level of soil nitrogen (Jain and Patriquinn, 1984).

In summary, there is reasonable evidence that this endophytic nitrogen fixation can attain a magnitude which is of definite ecological and economic significance. Additionally, it should be noted that these endophytes constitute a group of species which can tolerate adverse conditions of growth such as low temperature, or waterlogged and acid soils. Thus the group is in a position to fulfill a role of nitrogen providers under very varied circumstances.

Potential for future research

Extending endophytic nitrogen fixation to all cereal crops of economic significance may be tried by the following two methods

Genetic modification of host plant

- To avoid hypersensitive reactions of the host plant.
- To avoid production of phyto-alexins.
- To increase host compatibility to accommodate endophytic nitrogen fixers.
- To provide areas of low redox potential for the establishment of endophytes by means of diffusion barriers or leghaemoglobins.
- To make the membrane systems around the endosymbiont facilitate bi-directional transport of substances.
- To study *Nif*-gene transfer.

Genetic modification of the microorganisms

- To induce production of auxins such as 2,4-D, NAA etc.
- To produce cellulases and pectinases, which can degrade the cell walls.
- To make the endophytes more tolerant to oxygen concentrations.
- To increase the ability of endophytes to colonize both intra and inter-cellularly.
- To increase the span of microbial life in vegetative phase.
- To make the microorganisms use the host materials as energy source but without any phyto-pathogenic effect.
- To make microorganisms produce sufficient amounts of superoxide dismutase.
- Selection of promising strains possessing high nitrogenase activity.
- To screen germplasm for diazotrophic colonization.
- To investigate root morphogenesis in relation to invasion.
- To study in detail the interactive responses between flavonoids and nod-factor.
- To investigate the metabolic programming of cereals for attachment of microorganism and protection of nitrogenase from oxygen inactivation.

Conclusion

The ambitious goal of achieving nitrogen fixation in cereals naturally arouses controversy. However, despite the biological obstacles, there are strong and obvious grounds for achieving it based on environmental and economic considerations. This goal is also towards a more organically based agriculture. The progress made in this field of study since 1992 suggests that the research is on schedule to deliver positive outcomes in the medium term of 5-15 years.

Currently there are challenges with respect to factors such as whether the high numbers of bacteria found endophytically in seedlings (10^x cells g^{-1} fresh weight roots) can be maintained in larger plants and whether nitrogenase activity

will be adequate with the O_2 concentrations found in soil. Overcoming these obstacles will require significant genetic improvements of both host plant and bacteria. These improvements can be hastened by selections made in plants for superior genotypes favouring endophytic colonization of the plant.

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