EFFECT OF *Azorhizobium caulinodans* AND PHYTOHORMONES ON PARANODULATION, ESTABLISHMENT AND YIELD OF RICE

Thesis

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

MASTER OF SCIENCE
in
MICROBIOLOGY
(Minor Subject : Biochemistry)

BY

Manu Goyal
(L-2002-BS-174-M)

Department of Microbiology
College of Basic Sciences and Humanities
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Gift

CERTIFICATE I

This is to certify that the thesis entitled **"Effect of *Azorhizobium caulinodans* and phytohormones on paranodulation, establishment and yield of rice"** submitted for the degree of **Master of Science** in the subject of **Microbiology (Minor subject : Biochemistry)** of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Ms. Manu Goyal (L-2002-BS-174-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Thesis

17.12.02

Major Advisor
(Dr R.P. Gupta)
Professor of Microbiology
Department of Microbiology,
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15105

CERTIFICATE II

This is to certify that the thesis entitled, "**Effect of *Azorhizobium caulinodans* and phytohormones on paranodulation, establishment and yield of rice**" submitted by **Ms. Manu Goyal (L-2002-BS-174-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **Master of Science**, in the subject of **Microbiology (Minor Subject : Biochemistry)** has been approved by the Student's Advisory Committee along with Head of the Department after an oral examination on the same.

Head of the Department
(Dr P.K. Khanna)

Major Advisor
(Dr R.P. Gupta)

Dean, Post-Graduate Studies
(Dr Darshan Singh)

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ABSTRACT

Attempts are being made to extend the symbiotic N_2 fixation to non legumes such as rice. Ten isolates of *A. caulinodans* isolated from root and stem nodulating leguminous green manure *Sesbania rostrata* possessed specific intrinsic antibiotic resistance spectra and nitrogenase activity with maximum *in vitro* nitrogenase activity recorded by SRR-1. SRR-1 and two reference strains along with phytohormones were used for induction of paranodules and nitrogen fixation in the roots of rice cv. Bas-386 and cv. PR-116 under laboratory as well as field conditions. The inoculation of *A. caulinodans* strains showed increase in root-shoot length, biomass, paranodule number, lateral rootlet number and nitrogenase activity using kinetin and gibberellic acid under laboratory conditions. Kinetin gave better results over GA_3. In the field trial, inoculation of strains resulted in significant increase in all the parameters studied at all stages of growth while the rice roots treated with 2,4-D along with cultures showed enhanced paranodulation nitrogenase activity, yield and yield parameters in both the varieties.

Key words : Paranodulation, cereals, *A. caulinodans*, phytohormones

17.12.04
Signature of Major Advisor

Manu Goyal
Signature of the Student

CONTENTS

Chapter I

INTRODUCTION

Nitrogen is the most critical nutrient for rice productivity that requires 1 kg of nitrogen to produce 15-20 kg of grains. Low land rice in tropics can use nitrogen available either naturally through BNF or from mineralisation in soil to produce 2-3 t of grain/hectare (Watanabe and Roger 1984). However, additional nitrogen must be supplied for higher yield. In modern agriculture the replenishment of soil nitrogen most commonly involves the extensive application of chemical fertilizers, an approach that suffers from several drawbacks including high costs, severe negative environmental impacts and aftermaths on soil texture and health.

Chemical nitrogen fertilizers being ecologically and economically expensive, interest in exploitation of alternative or supplementary nitrogen sources has been renewed to encourage sustainable agriculture (Shenoy *et al* 2001). Rice suffers from a mismatch of its nitrogen demand and its nitrogen supplied as chemical fertilizers, resulting in a 50-70% loss of the fertilizer applied (Reddy *et al* 1997).

Few agrotechnologies meet the criteria for sustainable agriculture development as neatly as the legume BNF (Gupta and Pandher 1996).

Asymbiotic associative or symbiotic biological nitrogen fixation (BNF) is a free and renewable resource which constitute an integral part of sustainable agro-ecosystems (Jensen and Nielsen 2003). Global terrestrial BNF is between 100 and 290 million tonnes of N $year^{-1}$ (Cleveland *et al* 1999), 40-48 million tonnes $year^{-1}$ of which is fixed by agricultural crops in fields (Galloway *et al* 1995 and Jenkinson 2001). 80% of stable BNF is a direct result of the symbiotic interaction of members of Rhizobiaceae and some actinomycetes with leguminous as well as certain non leguminous plants (de Bruijn *et al* 1995).

BNF derived nitrogen assumes significance in lowland system that provide about 80% of the world's rice. Conventional BNF systems of rice the free living/associative diarotrophs have low to moderate potential to supply N as N fixed outside the plant is subjected to loss and thus inefficiently utilised as compared with those of legumes under suitable conditions. BNF assembled in the rice plant enhance the N supply potential as fixed N would be available directly with little or no loss (Ladha *et al* 1998). Thus, it has become imperative to consider the unconventional systems to supply rice with N (Kannaiyan *et al* 2001). Extending legume-*Rhizobium* symbiosis to non legumes will significantly increase the amount of available nitrogen and thereby biomass of several cereal and other non-legume crops (Kalia and Gupta 2002). "Endophytic

BNF" inside the root of the rice plant reduce the fertilizer consumption in wetland rice in sustainable rice farming (Kannaiyan 1998).

Green manure crops can supply a substantial portion of the nitrogen required by rice. Recently, *Sesbania rostrata* has been found to be an excellent candidate for green manuring in lowland rice systems as it is fast growing, flood tolerant legume, supports nitrogen fixation under free living conditions and combined nitrogen and produce stem as well as root nodules (Kannaiyan and Kalidurai 1995). Dreyfus *et al* (1984) reported that stem nodulating rhizobia in *Sesbania rostrata* is *Azorhizobium caulinodans* that invades the plants by a specialised process called crack entry that involves entry at region of new emerging lateral roots.

The bacterium *A. caulinodans* is type species that fixes nitrogen under free-living conditions and also tolerate 12 µM of dissolved oxygen (Gough *et al* 1997b). *A. caulinodans* tolerate flooded condition and produces the pectic and cellulolytic enzymes with good penetration capacity and fixes nitrogen in rice root system at microaerophilic condition (2-3% O_2 level) (Kannaiyan 1998).

Non legume nodulation could be achieved either by inoculation of natural endophytic diazotrophs or by hydrolytic enzyme treatment along with polyethylene glycol/calcium chloride treatment (Al-Mallah *et al* 1989a,b) or by use of growth regulators as auxins (2,4-D, NAA), cytokinin (Nie 1993) or by use of signal phenolic compounds such as

flavonoids, isoflavones and flavanols (Jain and Jain 1998). These treatments abolish complex interactive signal reactions needed by both bacteria and host for initiation of lateral root formation resulting in formation of effective nodule-like structures (paranodules) with detectable nitrogenase activity.

The root systems of rice, wheat and maize showed infection by entry through cracks with rhizobia isolated from stem nodules of *Aeschynomene* and *S. rostrata* and from *Parasponia* (Vij and Chopra 2000). Kennedy and Tchan (1992) reported root hypertrophies in non legumes by 2,4-D, NAA or hydrolytic enzymes treatment, invaded by diazotrophs that increase the nitrogen uptake by plant.

A. caulinodans strain ORS571 enters the roots of wheat and rice at the points of emergence of lateral root cracks (Webster *et al* 1997). *A. caulinodans* inoculation along with growth regulators as 2,4-D, NAA and kinetin increases growth parameters of rice, maize and cumbu due to increase of lateral rootlets (Amutha and Kannaiyan 1999). Buvana and Kannaiyan (2002) studied the effect of cell wall degrading enzyme mixture and NAA along with *A. caulinodans* on induction of paranodules in rice.

During the development of lateral root emergence a minute way is formed on both sides of lateral root by which *A. caulinodans* established deep colonization through lateral cracks, travels intercellularly in cortex

to colonize xylem of rice. Rolfe *et al* (1998) exhibited xylem elements as possible sites of nitrogen fixation by diazotrophs as they could provide low pO_2 and site for metabolic exchange necessary for nitrogen fixation. O'Callaghan *et al* (1997) showed xylem colonization of *Sesbania rostrata*. Gopalaswamy *et al* (2000) reported *A. caulinodans* xylem colonization in rice roots. Extensive colonization of xylem of *Arabidopsis thaliana* by *A. caulinodans* ORS571 was observed by Stone *et al* (2001).

Rice root associated nitrogen fixation through nodular structures in rice is like "minifertilizer" factory in rice root itself. The ability of this micro-organism to establish endophytically form effective symbiotic nitrogen fixation with rice need to be investigated with following objectives :

1. To study the effect of kinetin and gibberellic acid on paranodulation by *Azorhizobium caulinodans* through crack entry in rice roots.
2. To study the effect of *A. caulinodans* along with phytohormone (2,4-D) on nitrogen economy and yield of rice.

Chapter II

REVIEW OF LITERATURE

Cereals are the world's major source of food for human nutrition. Among these, rice is very important and represents the staple diet for more than two-fifths (2.4 billion) of world's population, making it the most important food crop of the developing world. As a result of green revolution, there was a tremendous change in the food grain production scenario and since then fertilizers have become the major input in world rice supply (Barker *et al* 1985). Global agriculture now relies heavily on N fertilizers costing agriculture more than US $ 45 billion per year (Ladha and Reddy 2000). The demand for N fertilizer in world agriculture is increasing at a rate approximately equivalent to the rate of increase in world population in about two per cent per annum. The present world population of 6 billion is expected to become more than 9 billion by the year 2050. At the same time, the arable land will decline due to the pressure of urbanization (Khush 1997). Thus, additional food is to be procured from lesser land in the future.

The availability of usable nitrogen in soil has always been a limiting factor in agriculture. Rice recovers about 30-40% of applied

nitrogen. Accordingly, the rice plant must absorb a large amount of nitrogen to produce higher yield. At current levels of N use efficiency, this would require approximately double N fertilizer currently used each year for rice production (Ladha and Reddy 2003). The worst part of this scenario is the low input efficiency of N fertilizer, decline in crop yield under continuous cropping and severe environmental impacts like nitrate pollution, acidification of soils and emission of greenhouse gases such as ammonia and nitrous oxide (Shenoy *et al* 2001).

Reducing the fertilizer nitrogen use while maintaining the native soil nitrogen resources and enhancing crop nitrogen output by biological nitrogen fixation is desirable from both environmental and economic perspectives. The role of BNF on nitrogen cycling, ammonia volatilisation, N_2O emission and NO_3 leaching suggests that BNF is less likely than fertilizers to cause losses during pre-cropping and cropping (Jensen and Nielsen 2003). Intrinsically, BNF symbolizes and explains sustainability as it is inexpensive, renewable does not pollute or may even provide a positive contribution to its own environment. The researchers thus considered BNF the most 'environmentally friendly' approach to supply N to agroecosystems. The major source of earth's fixed nitrogen is in the form of ammonia, amide, nitrite or nitrate from the biological origin, which is estimated to be around 175 million metric tonnes per year

with approximately 90 million metric tonnes fixed in soil (Peter and Burgess 1980).

The total nitrogen requirement for a good deep-water rice crop was estimated at 147 kg N hectare^{-1} (Rother *et al* 1988). Although, acquatic ecosystems are able to meet part of their nitrogen demand, the rest needs to be supplemented. The supplemented chemical fertilizers also has adverse effects, such as nitrate contamination of underground water and growth of non target acquatic weeds. Alternatively, farm yard manure, BNF, such as crop rotation with legumes and *Azolla*, also contribute toward usable nitrogen in soil for agriculture purposes. However, these practices are quite uneconomical, laborious and also not self sufficient enough to provide the total nitrogen requirement to the rice crop (Dey and Datta 2002). In order to make rice cultivation sustainable and less dependent on fertilizer nitrogen, there is a need to use diazotrophic bacteria that can make biologically fixed nitrogen available for the growth of rice plants (Ladha *et al* 1997).

The endosymbiotic association that operates between legume (also few non legumes) and soil bacteria, commonly grouped as rhizobia, is an efficient process where rate of fixation is very high and all of it is retained within the plant without being lost to the environment. On a global scale, rhizobia-legume symbioses provide a quantity of fixed

nitrogen equivalent to that produced by the entire chemical fertilizer industry.

Scientists have been keen to explore the feasibility of endowing rice plant with endosymbiotic nitrogen fixation capability which could be attained by :-

1. Increasing the attachment interaction of rice roots and the submerged shoots with the free-living and plant adhered diazotrophs combinely constituting the rhizocoenosis of rice.
2. Induced nodulation of rice roots in presence of certain phytohormones and signal compounds in presence of certain specific rhizobia and symbiotic nitrogen fixation.
3. Genetic engineering of rice genome by addition of leghaemoglobin gene of non legumes as *Parasponia* to rice and enhancing its capacity to inhabit diazotrophs (Wei *et al* 1998).

2.1 Indigenous BNF systems (Autochthonous)

The major BNF systems are cyanobacteria and photosynthetic bacteria that inhabit flood waters and soil surfaces, as well as heterotrophic bacteria in the root zone and free living in the soil (Ladha and Reddy 2003). Cyanobacteria have low to moderate N fixing potential but the N fixed being outside the plant, is subjected to losses and also not immediately available to the plant (Reddy and Ladha 1995). *Azotobacter*, *Clostridium*, *Drexia* and *Beijerinckia* are pioneers to colonize rice

rhizosphere and histosphere and provides a positive contribution to ecosystems in nitrogen they fix (approximately 25-30 kg N ha^{-1} $crop^{-1}$) perhaps indirectly when they decompose or directly (Kennedy and Tchan 1992). *Azotobacter* inoculation results in increase in yield and yield attributes and nitrogen uptake in wheat (Singh *et al* 2000). Benign organisms such as free living diazotrophs, in addition to fixing atmospheric N, compete for space with pathogens, thereby reducing the diseases.

2.2 Exogenous BNF systems (Allochthonous)

BNF systems include *Azolla* that harbour symbiotic N_2-fixing cyanobacteria and acquatic legumes like *Sesbania* and *Aeschynomene* species that form symbioses with heterotrophic and phototrophic rhizobia (Ladha and Reddy 2003). The symbiotic association of endophytic BGA *Anabaena azollae* with water fern *Azolla* can fix 30-60 kg N ha^{-1} per rice yield (Kannaiyan and Gopalaswamy 2002). *Azolla* considerably increases growth and grain yield of rice cv. Riho (Badaway *et al* 1998). *Azolla* mineralises rapidly and its nitrogen is made available to the rice in a very short period. Rainfed rice environments that are prone to waterlogging are most suitable for growing green manure legumes (Ladha and Garrity 1994). In recent years, *Aeschynomene* sp. and *Sesbania rostrata* (Dreyfus and Dommergues 1981) growing as wild plants under waterlogged soils in Senegal (Africa) were recommended as potential nitrogen fixing

leguminous green manure plants which produce nodules on both roots as well as stem by a specific *Rhizobium* viz. *Azorhizobium caulinodans*. *S. rostrata* could add substantially higher amounts of nitrogen than is required by rice crop (Ladha *et al* 1988). In India, Kannaiyan *et al* (1987) reported that *S. rostrata* contributes 40-60 kg N ha^{-1} for rice crop per season and significantly increased the grain yield of rice.

Stem nodulation is an advantage over root nodulation (Ladha *et al* 1989) as nodules are produced on the above ground part of the plant where competition with other rhizobia was negligible. *S. rostrata* is unique to form green photosynthetic stem nodules on pre-determined nodulation sites of lateral root primordium emergence, the region where cracks appear in root epidermis exposing cortical cells leading to crack entry of a specific bacteria (rhizobia) viz. *Azorhizobium caulinodans* (Balasubramani and Kannaiyan 1992a). 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.

Robertson and Alexander (1994) suggested that rain and wind blown soils are the means of transmitting the bacterium *A. caulinodans*, thus favouring stem nodulation. The epiphytic occurrence of *A. caulinodans* in *S. rostrata* plant parts including leaves, stem, flowers, pistil was reported (Balasubramani and Kannaiyan 1991 and

Balasubramani *et al* 1992). *A. caulinodans* was also isolated from the various parts of ants, mealy bugs and beetles (Kannaiyan 1997).

Balasubramani *et al* (1992) have reported that nitrogenase activity was significantly higher in stem nodules than in root nodules of *S. rostrata*. *S. rostrata* stem nodule recorded nitrogenase activity of about 600 μM C_2H_4 h^{-1} $plant^{-1}$ in low land rice condition (Dreyfus *et al* 1984). Manguiat *et al* (1987) stated that flooding reduced nitrogen fixing activity of root nodules due to root decay but not stem nodules. *S. rostrata* callus inoculated with *A. caulinodans* showed nitrogenase activity under *in vitro* conditions. The movement of *A. caulinodans* within *S. rostrata* was studied and established by isolation from different plant parts of inoculated *S. rostrata* (Chitra ad Kannaiyan 1994). They also reported the presence of *A. caulinodans* both in the rice rhizoplane region and in rice soil.

2.3 Endogenous BNF systems (in planta)

Bennett and Ladha (1992) highlighted the advantage of self-sustaining rice and provided a critical assessment of the feasibility of N fixation in rice. Several approaches toward developing rice capable of fixing N_2 are now being considered such as establishment of endophytic symbioses, development of legume-like nodulation, as well as introduction and expression of nitrogen fixation (*nif*) genes into rice plants (Ladha and Reddy 2000).

2.3.1 Rice-endophytic diazotroph associations : Non nodular associations

Roots of healthy plants grown in natural soil eventually develop a continuum of root-associated micro-organisms extending from the rhizosphere to the rhizoplane and even deeper into the epidermis, cortex, endodermis and vascular system. Many diazotrophic bacteria were isolated over the years from rhizosphere soil or the rhizoplane of a big variety of non-leguminous plants (Dobereiner 1992). Bacillo-Jimenez *et al* (2003) indicated that rice exudates may induce a higher chemotactic response for endophytic bacteria than for other strains present in the rhizosphere. Endophytic diazotrophs have been proposed to be responsible for the supply of biologically fixed N to their host plant (Boddey *et al* 1995). By inhabating the interior of the plants, these bacteria are thought to (1) avoid competition with bacteria of the rhizosphere and (2) derive nutrients directly from the host plants (James and Olivares 1998). In return, the plant interior (which is low in O_2 and relatively high in carbon) provides an environment conducive to N fixation allowing the bacteria to efficiently transfer fixed N products to the host (Sprent and James 1995). Quispel (1991) had suggested that only in endophytic systems are the prerequisites for effective nitrogen fixation likely to be fulfilled in interactions between non legumes and diazotrophic bacteria. *Gluconacetobacter diazotrophicus* (syn. *Acetobacter*

diazotrophicus) a sugarcane associate represents a model system for monocot-diazotrophic associations (Muthukumarasamy *et al* 2002) which contribute to 150 kg N ha^{-1} $year^{-1}$ (Boddey *et al* 1995). A new genus *Azoarcus* inhabits the roots of Kallar grass (*Leptochloa fusca*). *Azoarcus* strain BH72 invade roots and colonize the cortex cells of roots as well as stem bases and shoot of gonotobiotically grown rice seedlings cv. IR36 (Hurek *et al* 1994). Dobereiner *et al* (1993) speculated that endophytic diazotrophs in certain rice genotypes may, in fact, be responsible for the substantial contributions of BNF. If endophytic diazotrophs are partly or wholly responsible for BNF in sugarcane and Kallar grass, it is possible that rice varieties that show significant heterotrophic BNF (Malarvizhi and Ladha 1999, Shrestha and Ladha 1996) also obtained fixed N from bacteria living within their tissues.

Some or all the endophytes may be responsible for supplying plants with fixed N. *Pseudomonas diazotrophicus* colonizes the root systems of sugarcane (Li and Mac Rae 1991) forming 80% of total bacterial population associated with rice roots (Watanabe *et al* 1987). *Azospirillum brasilense* penetrate the roots of graminaceous plant species, grow in rhizosphere and intercellularly (Christiansen-Weniger and Van Veen 1991). Perg Gerk *et al* (2000) reported the mutants with enhanced nitrogenase activity in hydroponic *Azospirillum brasilense*-wheat associations. *Azospirillum* inoculation in wheat results in significant

promotion of plant growth (Didonet and Magalhaes 1993) and thus being recommended as biofertilizer for wheat (Sandhu 2001). *Azospirillum* inoculation increase nitrogen content of rice (Nayak *et al* 1986).

Inoculation experiments with the endophytes *Serratia marcescens* and *Herbaspirillum* sp. in non-sterilised soil under greenhouse conditions have shown that they can be readily introduced into the rice plant by application of bacterial cultures on seeds or roots prior to germination or planting (Gyaneshwar *et al* 2001). *Herbaspirillum* sp. when inoculated into rice seedlings resulted in 40% increase in total plant N (Dobereiner *et al* 1993). James *et al* (2002) reported that *H. seroepdicae* infect rice and result in increased $^{15}N_2$ incorporation. *Alcaligens faecalis* invades rice roots (You and Zhou 1989), *Klebsiella pneumoniae* form association with root system of sugarcane (Li and Mac Rae 1991), *Klebsiella oxytoca* and *Enterobacter cloacae* with rice (Fuji *et al* 1987), *Burkholderia sp.* with rice (Baldani *et al* 2001). These microbes have various mode of action which include fixing N, increasing the availability of nutrients in the rhizosphere, positively influencing root growth morphology and promoting other beneficial plant-microbe symbioses (Vessey 2003).

2.3.2 Rice – rhizobia symbioses : Nodular associations

Rhizobia associate with the roots of nonlegumes without forming true nodules (Yanni *et al* 1997), but their populations decrease in number in the absence of legume host plants (Chabot *et al* 1996). Direct growth

promotion of non legumes by rhizobia has also been reported (Noel *et al* 1996). The possibility of extending host range of rhizobia to non-legumes was encouraged with the discovery of a non-legume in the family Ulmaceae (but not a monocotyledon) *Parasponia andersonii*, that naturally forms nodules with *Rhizobium* (Trinick 1979) and that *Rhizobium parasponium* RP501 and *Bradyrhizobium* CP283 induce nodulation in oilseed rape (Cocking *et al* 1992). Photosynthetic *Bradyrhizobium* ORS278 which usually induce nitrogen fixing nodules on stems and roots of acquatic legume *Aeschynomene sensitiva* form natural endophytic association with the wild rice species *O. breviligulata* (Chaintreuil *et al* 2000). Rhizobia produce chemical molecules that can influence plant development, including phytohormones, lipo-chitoligosaccharide Nod factors, lumichrome, riboflavin and H_2 evolved by nitrogenase (Dakora 2003). *R. leguminosarum* bv. *trifolii* promote the vegetative growth and grain yield of Egyptian rice varieties grown in rotation with the legume *Trifolium alexandrium* (Yanni *et al* 1997). Several rhizobial strains from *Sesbania* and *Aeschynomene* invade emerging lateral roots by crack entry and induce the formation of short thick lateral roots (STLR) in rice (Ladha *et al* 1996) and wheat seedlings (Cocking *et al* 1993).

Biswas *et al* (2000a) have observed increase in uptake of nitrogen, phosphorus and potassium by 10-28% upon rhizobial inoculation in low

land rice fields. Biswas *et al* (2000b) reported that *Rhizobium* inoculation influences seedling vigor and yield of rice. Inoculation responses to *Rhizobium leguminosarum* bv. *trifolii* E11 and *Rhizobium* sp. IRBG74 stimulated early rice growth, increased grain and straw yield at maturity. A 20% increase in shoot growth and grain yield of *O. breviligulata* grown in green house was observed upon inoculation with one endophytic strain and one *Aeschynomene* photosynthetic strain. Rhizobial inoculation increases grain yield by 16% and photosynthetic rate by 12% in rice (Peng *et al* 2002).

2.3.3 *Azorhizobium caulinodans*

2.3.3.1 Occurrence

Azorhizobium is a usual member of family Rhizobiaceae that include *Mesorhizobium*, *Sinrhizobium*, *Rhizobium*, *Allorhizobium* collectively called Rhizobia. Legume-rhizobial interactions culminate in the formation of specialised structures called nodules, resulting in considerable assimilation of fixed nitrogen. Generally, nitrogen fixing nodules are formed on roots of host legumes but in certain plants stem nodulation is also reported. Stem nodulating rhizobia include both fast (3-4hr generation time) and slow growers (10hr generation time) (Balasubramani and Kannaiyan 1990). The *Aeschynomene* nodulating rhizobia belong to slow growing group (Carroll 1934). Johnson and Allen (1952) concluded that rhizobia of most *Sesbania* sp. belong to fast

growing group. Dreyfus *et al* (1984) reported that *S. rostrata* was associated with both fast and slow growing rhizobial strains those infecting stem and root and those infecting root alone.

S. rostrata was associated with two potential strains *Rhizobium*, viz., the strain ORS571 that fixes atmospheric nitrogen in culture and grow at expense of the fixed nitrogen and nodulates both roots and stem of *S. rostrata* and other that nodulates root and does not have a capacity to fix nitrogen in a defined media (Dreyfus *et al* 1985). The root nodulating strain belonged to genus *Rhizobium* while root and stem nodulating strain ORS571 belonged to *Rhodopseudomonas palustris* (Jarvis *et al* 1986). These results were based on deoxy ribonucleic acid (DNA)-ribosome ribonucleic (rRNA) hybridizations. The exact taxonomical position of *Azorhizobium caulinodans*, was determined by a numerical analysis of phenotypic characters, comparative protein gel electrophoresis and DNA-DNA and DNA-rRNA hybridizations (Dreyfus *et al* 1988). The results led to the proposal of a new genus viz. *Azorhizobium caulinodans* containing a single species *A. caulinodans*. Recently 16S-23S ribosomal DNA intergenic space-targeted PCR allows specific detection of *Bradyrhizobium* and *Rhizobium* strains colonizing rice (*Oryza sativa*) roots (Tan *et al* 2001).

The closest relative of this organism is *Xanthobacter* though *Azorhizobium* strains are both phenotypically and genotypically different

from this genus. *Azorhizobium* and other stem/root nodulating rhizobia of *S. nostrata* showed G + C content ranging from 65-68%. de Bruijn *et al* (1988) and Fischer (1994) studied the molecular genetics of nitrogen fixation of *A. caulinodans* ORS571. Hydrogenase negative mutants of *A. caulinodans* ORS571 were isolated and characterized by de Vries *et al* (1988). The nitrogen fixation and hydrogen metabolism in relation to dissolved oxygen tension in chemostat cultures of wild type and Hup mutant of *A. caulinodans* was studied by Boogerd *et al* (1994). O_2 sensitive mutant of *A. caulinodans* with impaired N fixation ability exhibited a defective uptake hydrogenase during symbiosis (Das and Lodha 1998).

2.3.3.2 Morphology

Kalidurai and Kannaiyan (1988) studied the morphology and biochemical reaction. Balasubramani and Kannaiyan (1992b) reported *in vitro* screening media for stem and root azorhizobial isolates of *S. rostrata*. The cells of *A. caulinodans* are gram negative, small rods (0.5-0.6 µm by 0.5-2.5 µm) and motile. The cells have peritichous flagella on solid medium and one lateral flagellum in liquid medium (Dreyfus *et al* 1988). The colonies on Yeast Extract Mannitol Agar appear circular creamy, viscid and butyrous, fix N in microaerophilic conditions. Rodriguez *et al* (1987) have shown that the fast growers mostly grew as large, gummy, convex, viscid colonies and slow growers possess small

flat to small opaque or translucent colonies. The bacteria were found to be able to utilise glucose, mannitol, sucrose, maltose and lactose (Nael and Walker 1935) and *Azorhizobium* can thrive well in the presence of inorganic nitrogen while free living and organic acids such as lactate and succinate are the favourite carbon substrates.

The ability to form bacteriochlorophyll (Bchl) and photosynthetic reaction centre appears to be a fascinating and novel feature of the stem-nodulating microsymbiont. The cortical cells around stem nodules are capable of carrying out photosynthesis as they harbour chloroplasts and are so called "self energy generating systems" (Fleischmann 1991). Ladha *et al* (1990) reported that *Azorhizobium* contains bacteriophyll and photosynthetic reaction centre. The stem nodulating rhizobia produce pigment only under aerobic/light conditions and failed to grow anearobically.

2.3.3.3 Nitrogen fixation under free living conditions

Rhizobia generally show nitrogen fixation under symbiotic conditions in host but Ludwig (1988) reported the possible role of nitrogen fixation of different rhizobial isolates under free-living conditions. Ramasamy and Bal (1986) reported a limited number of rhizobia of genera *Bradyrhizobium* showing free-living nitrogenase activity. Alazard (1998) observed that nitrogen fixed by *Bradyrhizobium*

strain CB756 under free-living conditions was 10 times lower than that by *A. caulinodans* ORS571.

Dreyfus *et al* (1988) have reported that root and stem nodulating strain could grow on N under microaerobic conditions whereas no growth could be detected with *Rhizobium* and *Bradyrhizobium* under similar conditions. The nitrogenase activity was around 30 nM of C_2H_2 produced mg^{-1} protein min^{-1} in case of *A. caulinodans* while it was only 5-10 nM of C_2H_2 produced mg^{-1} protein min^{-1} in case of *Rhizobium* and *B. japonicum* strains. The nitrogenase activity of rhizobial strain on defined media depends on provision of suitable carbon source (Pagan *et al* 1975). *In vitro* nitrogenase activity was recorded relatively more in arabinose and mannitol followed by arabinose and sucrose in semisolid agar medium than solid agar medium (Kannaiyan and Kalidurai 1995).

Dreyfus *et al* (1983) reported that the *Rhizobium* ORS571 express very high nitrogenase activity under completely nitrogen free condition. The strain ORS407 exhibited poor nitrogenase activity on agar cultures (5-10 nM C_2H_4/h/mg protein). Kalidurai and Kannaiyan (1991) reported that both stem and root isolates of *A. caulinodans* could tolerate combined nitrogen at 2.5ppm and fix N.

Free living *Rhizobium* sp. fixes nitrogen in liquid medium, under low oxygen tension (Tjepkema and Evans 1975). Gebhardt *et al* (1984) reported higher tolerance of *Azorhizobium* (upto 9 μ moles) to oxygen.

Urban *et al* (1986) suggested nitrogen fixation by strain ORS571 at high oxygen concentration (3%) and temperature (37°C) without differentiation into bacteroids (Kitts and Ludwig 1994).

2.3.3.4 Nodulation by *A. caulinodans*

The discovery of first known rhizobial symbiosis with a non legume (*Parasponia – Rhizobium* symbiosis) by Trinick (1988) provided added impetus to extension of BNF to non legume crops (Davey *et al* 1997).

A new approach to the nodulation of non legume crops by rhizobia was the discovery that cell wall at the tip of the root hairs could be enzymatically removed from a wide range of both legume and non legumes including cereals. The removal of apical cell wall by a mixture of cellulase and pectinase expose the plasma membrane for the entry of the *Rhizobium* (Al-Mallah *et al* 1989b) and alter the specificity of rhizobia for nodulation (Cocking *et al* 1994). Nodulation in non legumes has also been achieved at low frequency by applying rhizobia to enzyme treated roots of rice, maize and oil seed. Omission of enzyme treatment to seedlings resulted in formation of no such structure (Cocking *et al* 1993). *B. parasponium*, *Aeschynomene* bacteria and *Rhizobium leguminosarum* bv. *trifolii* and *loti* are widely used rhizobia for paranodulation via hydrolytic enzyme treatment (Cocking *et al* 1990). The treatment with cell wall hydrolyzing enzyme enabled normally excluded *Rhizobium loti* to

infect *Trifolium repens* and form nitrogen fixing nodules (Cocking *et al* 1992).

Jing *et al* (1990b) reported the induction of pseudonodules on barley roots by *Rhizobium astragali* under a permanent magnetic field. A frequency induction of infected nodule like structures on rice roots by *Sesbania* rhizobia has also been reported (Li *et al* 1991). In fact, a positive effect of rhizobial infection of rice paranodules was observed by the acetylene reduction activity and the yield.

Azorhizobium caulinodans enter the plant intercellularly between adjacent cells of epidermis, probably as a consequence of their ability to secrete cellulases and pectinases (Cocking *et al* 1994). 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 generally fix N in legume nodules (Kennedy *et al* 1997). *A. caualinodans* strain ORS571 enters the roots of wheat and rice at the points of emergence of lateral roots (lateral root cracks) (Reddy *et al* 1997, Webster *et al* 1997). Sabry *et al* (1997) showed that wheat grown in pots and inoculated repeatedly with *A. caulinodans* colonized root tissues at the points of the emerging lateral rootlets and exhibited high levels of acetylene reduction activity. Cocking *et al* (1995) have clearly shown that inoculation of *A. caulinodans* increased the lateral root formation which attracted the free-living bacteria for both colonization

which inturn increased the growth and biomass production of the plants. The symbiont *A. caulinodans* ORS571 upon inoculation, stimulated the lateral root development as well as formation of nodular like structures in the roots of rice (Kannaiyan *et al* 2001).

Kumar *et al* (1996) 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. Senthil Kumar (2000) reported the effect of naringenin, enzyme mixture and *A. caulinodans* on induction of paranodules in rice and maize. The seedlings treated with naringenin, enzyme mixture and *A. caulinodans* reported maximum number of lateral roolets, rhizoplane population, nodule like structures and nitrogenase activity. The effect of flavonoid on root morphology of different rice varieties was studied and found that flavonoid naringenin at 5×10^{-3} M concentration significantly stimulated the lateral root formation in rice varieties (Gopalaswamy *et al* 2000). Gough *et al* (1997a) reported the lateral root crack colonization of *Arabidopsis thaliana* by ORS571 being stimulated by naringenin which is also nod factor independent. Internal colonization of lateral roots were also shown to be nod-factor and *nod D* independent by Webster *et al* (1998). The beneficial plant growth promoting association of *Rhizobium leguminosarum* bv. *trifolii* with rice roots (Yanni *et al* 2001) is particularly relevant to assessments of whether

rhizobia can fix N endosymbiotically in cereals. It was concluded that the benefits of this association leading to greater production of vegetative and reproductive biomass more likely involved rhizobial nodulation of the root architecture for more efficient acquistion of certain soil nutrients rather than BNF.

Induction of nodule formation by rhizobia is controlled by cascade of signal exchange reactions between two partners. Plants secrete phenolic compounds, flavonoids, that triggers induction of a set of bacterial genes collectively known as *nod* or *nol* genes involved in synthesis of specific lipochito-oligosaccharides of varying lengths (Fischer and Long 1992) carrying different side groups and substitutes that play major roles in conferring host specificity (Denarie and Cullimore 1993, Vijn *et al* 1993). These Nod factors further induce various plant responses, including root-hair deformation, cortical cell division, pseudonodule and nodule formation (Cullimore *et al* 2001, Perret *et al* 2000). Broughton *et al* (2003) reported that Nod factors which possess hormone – like properties, are key determinants in nodulation and allow rhizobia to enter the plant.

Azorhizobial nod factor consists of lipochitooligosaccharide chain as core which contains a trimer to pentamer of β,1-4-linked N-acetyl glucosamine (Glc NAC) residues and a fatty acyl moiety that replaces Glc NAC group at non reducing end. This structure is common to all nod factors independent of their origin. However, the type of modification, the

length of sugar chain and nature of fatty acid differ from strain to strain (Mergaert *et al* 1995). Nod factors of *A. caulinodans* ORS571 are pentamers carrying a vaccenoyl or strearoyl chain. On the non reducing end, the oligosaccharide is modified with a N-methyl and O-carbamoyl group and the reducing end is branched with a D-arabinosyl sugar.

2.3.4 Induced nodulation by Phytohormones

Besides the involvement of plant hormones in legume nodule development, it is likely that plant hormones also play important role in nodulation of non legumes (Hirsch *et al* 1997). Various genetic and non genetic approaches are being used to induce nodule like structures on roots of rice and other cereals. Tchan and Kennedy (1989) and Ridge *et al* (1992) reported the stimulating effect of auxins such as 2,4-D in induction of nodule like structures called 'paranodules'. The induction of paranodules by rhizobia and free living diazotrophs in combination with the growth regulator 2,4-D results in greater potential and significance to achieve nitrogen fixation in cereal crops (Bender *et al* 1990 and Tchan *et al* 1991). Many plant species including some monocots develop paranodules in response to application of 2,4-D (Kennedy and Tchan 1992).

Shizhen and Donguwai (1994) and Zhiguo *et al* (1994) reported that colonization and N fixation by *A. caulinodans* ORS571 in the 2,4-D induced paranodules was less sensitive to oxygen than in non

paranodulating roots. Yu and Kennedy (1995) reported colonization and N fixation by *A. caulinodans* ORS571 in wheat.

Chen (1993) and Chen *et al* (1992) reported the nitrogen fixation by *Azospirillum brasilense* and *A. caulinodans* in paranodules induced on wheat roots. Nie (1983, 1989) reported that 2,4-D induced paranodules were inhabited by diazotrophs. Christiansen-Weniger and Vanderleyden (1994) described the colonization of paranodulated maize roots by ammonia – excreting mutant of *Azospirillum*, labelled with a gus fusion. Inoculation of *A. caulinodans* and growth regulator 2,4-D at 0.5, 1.5 and 3 ppm results in the formation of nodule like structures in roots of rice, maize and sorghum (Chandrasekar and Kannaiyan 1995). There was no nodule like structure formation when either *A. caulinodans* or 2,4-D alone was applied.

Nie (1993) reported nodule inducing effect of 2,4-D on roots of a large number of plant species including wheat. The exogenous application of 2,4-D to wheat and rice plants induced modified root out growths (MROs) that resulted from induction of meristems (Zeman *et al* 1992, Rolfe and Mclver 1994). Pan *et al* (1998) observed the inoculation of *R. sesbaniae* and *A. caulinodans* on 2,4-D treated rice roots gave maximum nitrogenase activity of 77.6 nM C_2H_4 $plant^{-1}$ day^{-1} with inoculation rate of 20-100%. This association was also found to promote growth of rice. Christiansen Weniger (1996) and Amutha and Kannaiyan (1999) reported

that rice seedlings developed nodule like structures along primary and secondary roots when treated with the auxin 2,4-D and *A. caulinodans*. The establishment of bacteria inside paranodule tissues around the tumour bases, suggest that tumour induction could be suitable method of endophytic establishment of bacteria in the roots of the graminaceous crops. Amutha and Kannaiyan (1998) revealed the stimulation of lateral root formation in rice, maize and cumbu upon inoculation with *A. caulinodans* in the presence of growth regulators 2,4-D, NAA and kinetin.

Akao *et al* (1991) reported induction of nodule like structures in non nodulating soybean T-201 by 2,4-D treatment in presence of rhizobia that developed an infective zone and exhibited nitrogen fixing activity. Treatment of the roots with 2,4-D produced short thickened lateral roots which showed better colonization by *Pantoea agglomerans gusA* tagged strain (Verma *et al* 2001). Recently, Kalia and Gupta (2002) reported the induction of paranodules on roots of rice cv. PR-115 by inoculation of locally isolated root and stem cultures of *A. caulinodans* and use of very low concentrations of phytohormones i.e. 2,4-D and NAA 0.5-3 and 3-7 ppm respectively.

Other phytohormones such as indole 3 acetic acid, napathalene acetic acid, indole butyric acid and cytokinin as kinetin also play a significant role in formation of nodule like structures (de Bruijn *et al* 1995). Reddy *et al* (1997) reported that IAA produced by rhizobia seems

to promote formation of "thick short lateral root" (TSLR) in rice. 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 and kinetin ranging from 1.0 to 7.0 ppm could induce the formation of nodular structures (Kannaiyan 1998). Inoculation of rice with *A. caulinodans* along with growth hormones 2,4-D or NAA at low concentrations induced rootlets, paranodules and nitrogenase activity (Amutha and Kannaiyan 1999). Buvana and Kannaiyan (1998, 2002) reported that the combination of cell degrading enzymes mixture, NAA with *A. caulinodans* induce more number of paranodules in rice. Total nitrogen content also increased in treated plants compared to uninoculated control.

Dehio and de Bruijn (1992) reported that cytokinin plays an important role in signalling and is required for nodulation since it determines the early nodulin gene *enod 2* expression and promote nodulation deficient rhizobia (lacking common nod genes) to induce nodulation in plants. Amutha and Kannaiyan (2000) reported that increased concentration of kinetin with inoculation of *A. caulinodans* increased the number of nodule like structures in the roots of young rice seedlings. The growth regulator kinetin was used at 3.0, 5.0 and 7.0 ppm concentration and the level above 3.0 ppm was found to be inhibitory to the seedlings by recording decreased root and shoot growth, total biomass

and lateral rootlets. However, there is no nodule like structure formation in rice roots when *A. caulinodans* and growth regulator kinetin was treated in the seedlings separately.

Nodulation is achieved as an intricate interplay of cytokinins and auxins as cytokinins applied exogenously to seedling roots or excised roots repress lateral root formation whereas exogenous auxin stimulates lateral root development in seedling roots, excised roots and roots of mature plants (Torrey 1986). Several early nodulin genes (*ENOD*) have been found to be induced by cytokinin-*ENOD2*, *ENOD12A* and *ENOD40* (Hirsch and Fang 1994). The increase in transcript accumulation for these three early nodulin genes reflect the endogenous status of cytokinin in the inoculated root.

Although the actual functions of *enod* genes in nodule organogenesis and their homologs in rice are not yet clear, *enod40* has been proposed to play a pivotal role in the initiation of nodule formation (Charon *et al* 1997). Hirsch *et al* (1997) reported that the cytokinin benzylaminopurine (BAP) at concentrations ranging from 10^{-8} to 10^{-5} induced *MsENOD40* and *MsENOD2* gene expression. This response appeared to be specific to cytokinins, as the only other phytohormone which induced *MsENOD40* gene expression was kinetin. All of the cytokinins are adenine derivatives. Other nucleotide derivatives, such as

uridine (recently identified as the stele factor, Smith *et al* 1995) did not induce *MsENOD40* gene expression.

Nod factors and cytokinins induce similar inner cortical cell divisions, amyloplast deposition and expression of *enod12A*, a marker gene for cortical cell division (Bauer *et al* 1996, Ross *et al* 2004) as cytokinin also induces several Nod factor-inducible *enod* genes, including *enod40* (Fang and Hirsh 1998). Cytokinins and Nod factors may share elements of their signal transduction pathways in the inner cortex (Dey and Datta 2002).

2.3.5 Genetic approaches for paranodulation

Present era holds the crown of numerous discoveries regarding the molecular genetics of non legumes and the extension of legume-*Rhizobium* symbiosis to cereal crops. Plazinski *et al* (1985) successfully transferred genes associated with root hair curling (hac genes) from *Rhizobium trifolii* into a derivative of *R. trifolii* on plasmids pKT230 and pRK290. The root hair curling character could then be expressed on infection of rice seedlings. A similar transfer of genes from *R. trifolii* into *Azospirillum brasilense* on plasmid pVK-100 was also achieved.

Okon (1985) suggested that genetic modification of diazotrophs help them to acquire the genes for the synthesis of pectin degrading enzyme that could facilitate the root invasion. Jing *et al* (1990a) have described formation of root nodules using a mutant of *Rhizobium sebania*

(*A. caulinodans*). The structure of these nodules and their formation was claimed to be similar to those of legume nodules such as soybean, including the presence of leghaemoglobin. Egener *et al* (1998) showed that the interior of rice roots is conducive to the expression of nitrogenase genes by the endophytic diazotrophs.

Achieving N autotrophy by rice plant is a highly ambitious prospect, needing probably a decade to realize. It demands extensive engineering of nuclear and extra-nuclear genes (Dixon *et al* 1997). Several agronomically important genes have been introduced (alone or in combination) in different rice varieties from different ecosystems by protoplast, biolistic and *Agrobacterium*-mediated gene transfer methods (Datta *et al* 1999).

Bogusz *et al* (1988) transferred *Parasponia* leghaemoglobin gene in *Tremia tomentosa* and found by Southern and Western blotting that it was transcribed in roots of this non-nodulating plant. Wei *et al* (1998) obtained transgenic rice plants with *Parasponia* – leghaemoglobin gene which was found to be expressed on organ specific manner. The transformation was performed by pollen tube and gene gun technique on rice florets. The legume gene *Gs50* cloned from *Glycine soja* (Dey *et al* 1999a) and *enod40* from *Medicago trancatula* (Dey *et al* 1999b) has been successfully transferred into rice.

Transfer of N fixation (*nif*) gene into rice genome would involve transfer of at least 16 genes (Dixon *et al* 1997). In addition to ensuring that the *nif* genes would be expressed, nitrogenase must be protected from inactivation by oxygen. Of the three potential locations for introducing foreign genes – nucleus, the mitochondrian and the chloroplast – the 'chloroplast' appears to provide the most suitable environment for *nif* gene expression (Dixon *et al* 2000) resembling those of procaryotes. The *nif* encoded polypeptides are more stable when expressed in the chloroplast than cytosol (Ladha *et al* 1998). Dixon *et al* (1997) reported the formation of transgenic tobacco and *Chlamydomonas* by incorporation of *nif* genes and their expression in chloroplasts. Another recent interesting finding is that globins originally identified in legume nodules are now being found in genomes of both non nodulating legumes and non legumes (Appleby *et al* 1990).

2.4 Site of bacterial colonization in cereals

Depending on the host plant and the endophyte, biofertilizing PGPR may be found in all parts of plants (seeds, roots, stems, leaves, fruit etc.) (Vessey 2003). 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 without disease symptoms (Bell *et al* 1995,

Hallman *et al* 1997). 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, the tropical legume *Sesbania rostrata* (Vlachova *et al* 1987).

R. leguminosarum bv. *trifolii* preferentially colonized rice seedlings surfaces in clumps (Prayitno *et al* 1999). This occurred along grooves on rice root and colonize intercellularly in lateral roots formed on main roots near the culm region of seedlings. Wheat has been reported to be xylem colonized by azorhizobia and *Pantoea agglomerans*. Microscopic studies revealed that azorhizobia invaded intercellularly in cortical cells, xylem and root meristem. Youssef *et al* (1998) also observed similar phenomena. Recently An *et al* (2001) also reported the endophytes occupying the apoplastic intercellular spaces inside the host at the junction of lateral roots from primary roots (O'Callaghan *et al* 2001).

Moreover, it is 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). 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). Van

Alfen (1982) reported that such endophytes remain in xylem without impairing transpiration while on the contrary benefit plant by enhancing shoot growth (Kloepper *et al* 1992).

Elbeltagy *et al* (2001) by fluorescence stereomicroscopy reported that *Herbaspirillum* sp. strain B501 never entered the vascular tissue apparently preferring the apoplast of shoot tissues, colonized intercellular spaces. James *et al* (1997) have reported that *A. diazotrophicus*, *H. rubrisubalbicans* and *H. seropedicae* colonized xylem vessels of host plants. *Acetobacter* and *Azoarcus* sp. colonizes xylem vessels of sugarcane (Hurek *et al* 1994) and rice (Englehard 2000).

James *et al* (2001) also reported that endophytic PGPR reside in xylem vessel apoplast. James and Olivares (1998) considered xylem apoplast an ideal place for the location of endophytes (eg. constant supply of nutrients, circulation systems for beneficial products from the microsymbiont). However, Mc Cully (2001) postulated that xylem is a most unsuitable habitat for endophytes, and many plant pathogens bring about plant death by colonization of the xylem apoplast.

The finding that ORS571 colonizes xylem elements in *Sesbania rostrata* in addition to inducing and invading nodules in the root cortex (O'Callaghan *et al* 1997) provided the novel perspective that some rhizobia can exist as symbiont in nodules and as benign vascular endophytes. This raises the possibility that xylem colonization might

provide a non nodular niche for endosymbiotic nitrogen fixation in rice, wheat, maize, sorghum and other non legume crops (Cocking 2003). In agricultural terms, one of the most promising naturally occurring, non nodular xylem colonizing endophytic diazotrophic interaction is that of *Gluconacetobacter diazotrophicus* in sugarcane which provides major contribution to the high levels of nitrogen fixation (Boddey *et al* 2003).

The recent availability of various genetic tools and reporter genes based on fusions with *lacZ* and *gusA* is having a major impact on studying the xylem colonization (Gough *et al* 1996). Conditions within the intercellular spaces of colonized wheat were shown to be appropriate for nitrogen fixation by following the expression of *nifD – lacZ* fusion of ORS571 (Webster *et al* 1997). Cocking (2001) reported xylem colonization of tomato by *A. caulinodans* ORS571. Gopalaswamy *et al* (2000) demonstrated xylem colonization of *Oryza sativa* roots by *A. caulinodans* ORS571 (pXLGD4) being stimulated by flavonoid naringenin. Chaintreuil *et al* (2000) reported the intercellular invasion of epidermal cells of roots of *Oryza breviligulata*, rarely intracellular, by *lacZ* tagged mutant of *Bradyrhizobium* strain ORS278. Extensive colonization of the xylem of the *Arabidopsis thaliana* roots was observed by Stone *et al* (2001). Use of *lacZ* or *udiA* (GUS) tagged *A. caulinodans* strain ORS571 (Reddy *et al* 1997) and *Herbaspirillum seropedicae* strain

Z67 (Barraquieo *et al* 1997) shows colonization in the intercellular spaces in sub-epidermal and cortical cell zones of roots.

It should be noted that even though endophytic diazotrophs such as *A. caulinodans*, *Gluconacetobacter diazotrophicus*, *Azoarcus* sp., *Herbaspirillum* sp. and some strains of *Azospirillum* are able to colonize the root cortex, they do not establish themselves intracellularly within living cells. Rather, they colonize the apoplast i.e. the intercellular spaces, the xylem elements and other dead cells (James 2000). The bacteria colonize intercellularly almost exclusively, usually in the basal zone of the paranodules 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.

Chapter III

MATERIAL AND METHODS

The present investigation was carried out in laboratories of Department of Microbiology and experimental area of Department of Plant Breeding, Genetics and Biotechnology, Punjab Agricultural University, Ludhiana during 2002-2004.

3.1 GENERAL

3.1.1 Materials

3.1.1.1 Glasswares

Borosil glassware was used through out the study. Glassware was cleaned with chromic acid cleaning solution (100g $K_2Cr_2O_7$ in 1000 ml water + 500 ml conc. H_2SO_4) and washed with tap water. A distilled water rinse was given before use of each glassware.

3.1.1.2 Chemicals

Chemicals used in the study were obtained from Himedia Laboratories (India), Qualigens fine chemicals (India), Genei Pvt. (India) and Sigma chemicals (USA).

3.1.1.3 Seeds

Seeds of Rice (*Oryza sativa* cv. PR-116 and cv. Bas-386) were obtained from Department of Plant Breeding, Genetics and Biotechnology, Punjab Agricultural University, Ludhiana.

3.1.1.4 *Azorhizobium caulinodans* strains

The reference culture *A. caulinodans* S_1 used in the present study were obtained from previous studies done in Department of Microbiology, Punjab Agricultural University, Ludhiana. The reference culture *A. caulinodans* ZB-SK-5 was obtained from Dr. S. Kannaiyan, Tamil Nadu Agricultural University, Coimbatore (India).

3.2 METHODS

3.2.1 Isolation of *Azorhizobium caulinodans*

3.2.1.1 Isolation of bacteria from root and stem nodules of *Sesbania rostrata*

Single, healthy and well-developed nodules from root and stem of *Sesbania rostrata* were detached carefully and washed with tap water. Nodules were surface sterilized by 0.1 % mercuric chloride for 3 min and washed repeatedly with sterile distilled water. Further surface sterilization was done by treating nodules with 70% ethyl alcohol for 30 sec followed by sterile water washings. Nodules were crushed in the sterile water with a sterile glass rod. 0.1 ml of 10^{-5} dolution was spread on Yeast Extract Mannitol Agar (YEMA) medium.

The plates were incubated in the laboratory at room temperature (28±1°C) for 48 h. The distinct, creamy, slimy, viscid, butyrous and translucent colonies were selected that did not absorb the congored dye. These colonies were picked up and streaked repeatedly for purification on YEMA plates and finally subcultured and maintained in YEMA slants in the refrigerators.

Composition of YEMA (gl^{-1}) (Allen 1953)

Mannitol	10.0
Dipotassium hydrogenphosphate	0.5
Magnesium sulphate	0.1
Sodium chloride	0.1
Yeast extract	0.5
pH	7.0
Agar	20.0
Congo-red dye (0.25% in water)	10.0 ml

3.2.2 Characterization

The isolated *Azorhizobium* cultures were purified and confirmed by growing the isolates in Congo Red Yeast Extract Mannitol Agar medium. One ml of 48 h old diluted culture was poured and then YEMA was poured. Petri plates were incubated at 28±1°C. The fast growing isolates formed colonies in 48 h while slow growers appeared after 3 days. The growth pattern and phenotypic characters were recorded.

3.2.2.1 Ketolactose Test

Test isolates were grown on lactose medium in petriplates and incubated for 3-4 days at room temperature 28±1°C.

Composition of Lactose agar media (gl^{-1}) (Bernaertz and Deley 1963)

Lactose	10.0
Dipotassium hydrogenphosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Yeast extract	0.5
Distilled water	1000 ml
pH	7.0
Agar	20.0

Growth on petriplates was flooded with Benedict's reagent and the reaction was observed.

Composition of Benedict's reagent (gl^{-1})

Sodium citrate	17.3
Copper sulphate	17.3
Anhydrous sodium carbonate	10.0
Distilled water	1000 ml

3.2.2.2 Growth on Bromothymol blue Yeast Extract Mannitol Agar

A loopful of isolates when streaked on the medium and incubated at room temperature (28±1°C) produced a colour change in medium. This reaction was observed for the respective azorhizobial isolates.

Composition of BTB supplemented YEMA (gl^{-1}) (Norris 1965)

Mannitol	10.0
Potassium dihydrogenphosphate	0.8
Sodium chloride	0.2
Ferric chloride	0.01
Yeast extract	0.4
Bromothymol blue indicator (0.4%)	5 ml
pH	7.2
Agar	20.0

3.2.2.3 Nitrogenase activity (Hardy *et al* 1973)

A. caulinodans isolates were grown in YEM-broth for acetylene reduction assay. After 48 h incubation, cotton plugs of vials were replaced by subaseals and 10% of air was replaced with acetylene gas using a disposable sterile syringe. Vials were incubated for 24 h and the acetylene reduced or ethylene produced was determined by a gas chromatograph (Nucon gas chromatograph) with poropak–R column and a hydrogen flame–ionization detector (FID). The column was preheated at 100°C for activation. The injector and detector temperature was maintained at

100°C. One ml of sample gas was drawn from each vial and fed/injected into the column. The peak height was measured and nitrogenase activity was calculated by the formula.

$$\text{Nitrogenase Activity} = \frac{\text{K X No. of c.u. of sample X vol. of vial}}{\text{Incubation time X mg protein}}$$

The nitrogenase activity was expressed in nM of ethylene produced per h per mg of cell protein.

3.2.2.4 Estimation of Protein content (Lowry *et al* 1951)

Lowry method was used to estimate the protein content of *A.caulinodans* isolates.

Reagents

Solution A : 2% sodium carbonate in 0.1 N sodium hydroxide

Solution B : 0.5% copper sulphate in 1% of sodium potassium tartarate

Solution C : 50 ml of solution A + 1 ml of solution B.

Solution D : Folin-Ciocalteau mixed in equal volume of water before use.

Procedure

A. caulinodans isolates were grown in YEM–broth for 48 h and known volume of it was centrifuged at 10,000 rpm for 30 min. The culture suspension (0.5 ml) was taken in a test tube and volume made up to 1 ml

with distilled water. 5 ml of solution C was added to each tube and allowed to stand for 10 min. Then 0.5 ml of solution D was added and contents mixed thoroughly. Blank was run simultaneously by using 1 ml of distilled water instead of sample solution. The colour was read after 20 min at 520 nm in spectronic-20 calorimeter.

Standard curve

A standard curve for protein was prepared under the same conditions using bovine serum albumin at a concenration of 50-250 µg/ml. The protein content was expressed as mg g^{-1} dry weight of cells.

3.2.2.5 Antibiotic Resistance Spectra

All isolates of *A. caulinodans* were tested for antibiotic resistance pattern. Aqueous solutions of different antibiotics were made with varying concentrations. Ethanol works as solvent for erythromycin, and 1M NaOH for nalidixic acid. The stock solutions of 10 mg/ml were prepared in respective solvents and were sterilized by filtration through 0.2 µM disposable membrane filter assembly (Millipore-GX).

Preparation of Antibiotic testing media

Three different final working concentration of 10 µg/ml, 20 µg/ml and 100 µg/ml were made by adding 0.02 ml, 0.04 ml and 0.2 ml of stock solution to 20 ml of YEMA medium, in sterile petriplates. Antibiotic solutions were added using an adjustable 200 µl microlit micropipette

having disposable tips. Plates were moved clock and anti-clockwise to thoroughly mix the contents.

Inoculation

An arrow was marked at the bottom of petri plates containing the known antibiotic solution at known concentration. Test strains were streaked on the antibiotic containing YEMA media. Test cultures were previously grown in YEM broth and later ten fold diluted to get approximately 10^8 cells/ml. Appearance of the culture growth showed presence of resistance to a particular antibiotic.

Antibiotic Solutions

Stock solutions of 10 mg/ml were prepared of following six antibiotics:

Vancomycin	- Distilled water
Kanamycin	- Distilled water
Neomycin	- Distilled water
Polymyxin B	- Distilled water
Streptomycin	- Distilled water
Erythromycin	- 95 % ethanol
Nalidixic acid	- 1M NaOH

3.2.3 In vitro technique for raising rice seedlings

3.2.3.1 Seed sterilization

Rice seeds of cv. PR-116 and cv. Bas-386 variety were surface sterilized by treating with 0.1% (w/v) mercuric chloride for 3 min and washed 4 to 5 times with sterile distilled water. A 70% ethanol solution was used later and seeds were treated with it for 2-3 min followed by repeated washings with autoclaved water.

3.2.3.2 Seed Germination

Surface sterilized seeds were placed on petriplates having moistened filter paper. About 25 seeds were placed on filter paper in a single petriplate. These plates were kept in dark at room temperature (28°C) for germination.

3.2.3.3 Seedlings Transfer

Healthy, well-sprouted and uncontaminated seedlings or sprouted seeds were aseptically transferred in 20 x 2.5 cm tubes containing plant nutrient media. Seedlings were transferred with a long forceps sterilized by flaming with spirit on a Bunsen lamp.

3.2.3.4 Hydroponic System

It consisted of long tube (20 x 2.5 cm) having a double–M whatman filter paper no.1 wick. Thirty ml of nitrate free media was sterilized by autoclaving. Forty eight hour old culture was added (250 µl) into 30 ml

nutrient media after 7 days of seedling growth. Two phytohormones viz. Kinetin and Gibberellic acid were added after 2 days of culture inoculations.

3.2.3.5 Aseptic Conditions

The laminar flow cabinet was sterilized with UV light and its working table and sides were thoroughly cleaned with cotton soaked in spirit. All instruments were also sterilized with spirit and made red hot over flame before use. The hands were properly washed with soap and disinfected with spirit. All transfers and inoculations were carried out using a Bunsen burner. The rim and necks of tubes were flamed before transfer or inoculation.

Composition of Plant Nutrient media (Broughton and Dilworth 1970)

Stock solutions (g/50 ml)

Solution I :	$CaCl_2.2H_2O$	14.705
Solution II :	$KH_2PO_4.3H_2O$	6.805
Solution III :	$FeCl_3.3H_2O$	0.180
	$MgSO_4.7H_2O$	6.165
	K_2SO_4	4.350
	$MnSO_4.H_2O$	0.016
Solution IV :	H_3BO_3	0.012
	$ZnSO_4.7H_2O$	0.014
	$CuSO_4.5H_2O$	0.005
	$CoSO_4.7H_2O$	0.002
	$Na_2MoO_2.2H_2O$	0.0024

0.5 ml each of four solutions was added to 1000 ml of distilled water to prepare the plant nutrient media.

3.2.3.6 Phytohormones

Two phytohormones were added to rice seedling roots.

- Kinetin (synthetic cytokinin) : 0.1% stock solution prepared in 0.1N NaOH. 1.5 ppm was used as working concentration.
- Gibberellic acid : 0.1% stock solution prepared in 0.1 N NaOH. 3 ppm was used as working concentration.

3.2.4 Effect of inoculation of *A. caulinodans* strains and phytohormones on growth and paranodulation in rice var. PR-116 and var. Bas-386

3.2.4.1 Laboratory experiment

3.2.4.1.1 Effect of inoculation of *A. caulinodans* strains and kinetin on rice var. PR-116 and var. Bas-386

Pre-soaked rice seeds of var. PR-116 and var. Bas-386 were surface sterilized as given in 3.2.3.1. Repeated sterile water washings were given and seeds were placed on petriplates containing moist filter paper. Uncontaminated, sprouted seeds were transferred to hydroponic system as described above. One week later 250 µl of 48 h old *A. caulinodans* cultures i.e. SRR-1 (root isolate), reference strains S1 (stem isolate) and ZB-SK-5 were added to the tubes. After 2 days kinetin was added according to following treatments.

T_1 – Control (No culture and no Kinetin)

T_2 – Culture alone

T_3 – Kinetin alone

T_4 – Culture + Kinetin

Following parameters were observed at 15DAI (days after inoculation):

- Root length – Root length was taken using scale, from the root-shoot junction to the tip of the longest root of plant.
- Shoot length – Shoot length was taken from root-shoot junction to the meristem of plant using a scale.
- Fresh weight – Total fresh weight was taken on electronic balance.
- Dry weight – Plant samples were dried at 60°C for 48 h and weighed on an electronic balance.
- Lateral roots – Number of lateral roots emerged were counted.
- Paranodule number – Number of nodule - like structures appeared on rice roots were recorded.
- Nitrogenase activity – The nitrogenase activity was detected by acetylene reduction activity (ARA). The rice roots were washed thoroughly with tap water to wash away the media. These were blotted to remove drenching water and placed in 15 ml test tubes and closed with the rubber stoppers. 10% of air (1.5 ml) was taken out using a sterile disposable syringe and same amount of acetylene

was added/injected. Vials were incubated for 24 h at room temperature. 1.0 ml of the sample gas was injected in gas chromatograph. The number of chart units moved by indicator were recorded and nitrogenase activity was calculated in μM of C_2H_4 produced per plant per 24 hour.

- Establishment of *Azorhizobium caulinodans* – *A. caulinodans* isolates were re-isolated from rice roots by procedure given by Vincent (1970). Fresh rice roots were washed with distilled waster and surface sterilized by 0.1% mercuric chloride and 70% ethanol followed by sterile water washings. The roots were crushed in a test-tube using a sterile glass rod. The extract was streaked on YEMA medium and was incubated at 28±1°C for 48 h.

3.2.4.1.2 Effect of inoculation of *A. caulinodans* strains and gibberellic acid on rice var. PR-116 and var. Bas-386

Rice seeds of var. PR-116 and var. Bas-386 were soaked in distilled water for overnight and surface sterilized as given in 3.2.3.1. Uncontaminated germinated seeds were transferred aseptically to hydroponic system. Gibberellic acid was added as detailed below :

T_1 – Control (No culture and no GA_3)

T_2 – Culture alone

T_3 – Gibberellic acid alone

T_4 – Culture + gibberellic acid

Following growth parameters were observed at 15DAI:

- Root length
- Shoot length
- Fresh weight
- Dry weight
- Lateral roots
- Paranodule number
- Nitrogenase activity
- Establishment of *Azorhizobium caulinodans*

3.2.5 Effect of inoculation of *A. caulinodans* strains, 2,4-D and N fertilizer on nitrogen economy and yield of rice var. PR-116 and var. Bas-386

3.2.5.1 Field experiment

The field experiment was performed in Rice-B block fields of the Department of Plant Breeding, Genetics and Biotechnology, Punjab Agricultural University, Ludhiana. Forty-eight plots of size 3.30 m x 2.20 m of var. PR-116 and 3.30 m x 2.00 m of var. Bas-386 were prepared and ploughed before sowing to get rid of weeds. Nursery of rice var. PR-116 and var. Bas-386 was prepared. One month old rice seedlings were uprooted and then transplanted. Before transplantation rice seedlings were treated with 48 h old azorhizobial cultures grown in YEM broth at 28°C.

Seedling roots were treated with 3 strains (one root isolate SRR-1 and two reference strains i.e. *A. caulinodans* ZB-SK-5 and stem isolate S1) for 45 min. These roots were further treated with 2,4-D (0.1% stock solution in 0.1M NaOH) for 15 min. The treated rice seedlings were then transferred to field. The treatments were applied in RBD (factorial) manner with 3 replications. Urea as a source of nitrogen was applied to half of plots according to recommended dose of 67.5 g while 75% of recommended dose of 50 g was applied to rest half of plots after 15 days of transplantation (DAT). Again urea treatment was applied twice after 15 days interval to same plots. Then sampling was done at 15DAT to study following parameters :

- Root length – The length was measured in centimeters using a scale and measured from root-shoot junction to the tip of the longest root of plant.
- Shoot length – Shoot length was measured in centimeters using a scale from root-shoot junction to the meristem of plant.
- Root and shoot weight – Plants were carefully uprooted from the field and root and shoot fresh weight was taken. The weight was measured in grams with the help of a weighing balance.
- Dry weight – The plants were dried in an oven at 60°C for 48 h and root and shoot dry weight was taken on weighing balance.

- Nitrogenase activity – The roots with intact nodules were cut at the root-shoot junction and thoroughly washed-off attached soil with tap water. The roots were blotted to get rid off moisture. The roots were placed in vessel (150 ml) and stopped with rubber cork. 10% of the air of vessel (15 ml) was injected out with the help of a sterile syringe and same amount of acetylene was injected inside vessel. The roots were incubated for 24 h at room temperature. 1 ml of sample gas was injected in gas chromatograph and ARA was calculated as $\mu M\ pl^{-1}\ h^{-1}$.
- Competitiveness of inoculated strains – The rice roots were washed under tap water and surface sterilized by 0.1% chloramine-T solution for 3 min. The roots were then mashed in pestle mortar and extract was collected in a sterile test tube. The extract of roots was streaked on antibiotic supplemented YEMA medium. A combination of antibiotics for which the particular isolate was resistant was added in a single plate. The occurrence of colonies and there number gave the competitiveness. The unsterilized roots were also used in same manner to obtain the presence of isolates or native microbes in soil or rice rhizosphere.
- Yield – Rice yield was determined in quintal per hectare and weighed on a weighing balance.

- Paranodule number – Paranodules that appeared were counted with naked eyes and expressed as number per plant.
- Tiller number – Number of tillers per plant was counted.
- Grain weight – Thousand grains were counted and measured on weighing balance and expressed as gram per plant.

Chapter IV

RESULTS AND DISCUSSION

4.1 Isolation and characterization of *A. caulinodans* isolates from *S. rostrata*

4.1.1 Isolation of *A. caulinodans* from stem and root nodules of *S. rostrata*

Legume-rhizobial interactions mainly culminate in the formation of nitrogen fixing root nodules. However, stem nodulation, a fascinating biological system was reported by Dreyfus and Dommergues (1981) in *Sesbania rostrata*, a tropical legume. A total of ten isolates were obtained of which five were the root isolates – SRR-1, SRR-2, SRR-4, SRR-5 and SRR-6 while five were isolated from stem nodules viz. SRS-1, SRS-3, SRS-7, SRS-10, SRS-18 (Plate I).

4.1.2 Purification of reference *A. caulinodans* strains

The reference strains of *A. caulinodans* viz *A. caulinodans* ZB-SK-5 and stem isolate S_1 were obtained in single colonies and maintained on slants.

4.1.3 Growth characteristics

The growth characteristics of the stem and root isolates as well as the reference strains were studied (Table 1). The morphological studies

Table 1. Morphological and Biochemical Characteristics of *Azorhizobium* isolates

Azorhizobium isolates	Gram's reaction	Growth rate on YEMA	Colony character on Congo red supplemented YEMA	Growth reaction on BTB supplemented YEMA	Ketolactose test
SRS-1	-ve	Fast	SLR	Alkaline	-ve
SRS-3	-ve	Fast	SLR	Acidic	-ve
SRS-7	-ve	Fast	SLR	Acidic	-ve
SRS-10	-ve	Slow	SDR*	Alkaline	-ve
SRS-18	-ve	Slow	SDR*	Alkaline	-ve
SRR-1	-ve	Fast	SW*	Alkaline	-ve
SRR-2	-ve	Fast	SW	Alkaline	-ve
SRR-4	-ve	Slow	SLR	Alkaline	-ve
SRR-5	-ve	Fast	SW	Alkaline	-ve
SRR-6	-ve	Slow	SDR*	Alkaline	-ve
S1	-ve	Fast	SDR*	Alkaline	-ve
ZB-SK-5	-ve	Fast	SDR*	Alkaline	-ve

Slow - Growth after 2 days
Fast - Growth within 24 hrs

SW - Slimy white
SLR - Slimy light red
SDR - Slimy dark red

* Growth seen after 3 days

Plate I. **Isolated colonies of *Azorhizobium caulinodans* on YEMA medium supplemented with Congo red obtained from stem nodules of *Sesbania rostrata***

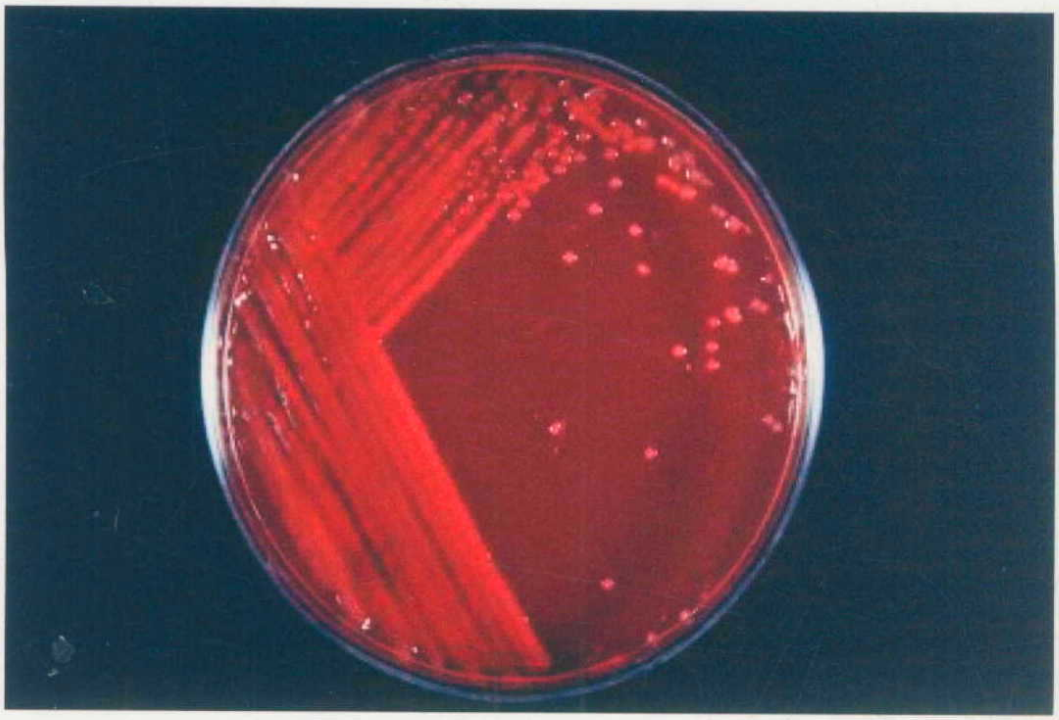

Plate I

revealed that the *A. caulinodans* cells are gram negative, motile rod cells having peritrichous flagella on solid medium. Among the *A. caulinodans* isolates both fast and slow growers were observed. Dreyfus *et al* (1988) reported that rhizobium isolates of *Sesbania rostrata* nodulate only root and were fast growers while *A. caulinodans* stem nodulating isolates of *S. rostrata* have both slow and fast growers which nodulate both root and stem. Among root and stem isolates SRR-4 and SRR-6, SRS-10 and SRS-18 were slow growers respectively. Both the reference cultures S_1 (stem isolate) and ZB-SK-5 were fast growers and alkali producers. All root isolates were found to be alkaline producers. While stem isolate SRS-3 and SRS-7 produced acid on BTB supplemented YEMA (Plate II). All the root and stem isolates did not produce yellow coloration in the lactose agar medium after flooding with Benedict's reagent confirming isolates to be *A. caulinodans*.

4.1.4 Antibiotic resistance spectra of isolates

The intrinsic antibiotic resistance spectra of isolates with seven different antibiotics viz. kanamycin, neomycin, vancomycin, erythromycin, streptomycin, polymyxin B and nalidixic acid were studied (Table 2). Each antibiotic was tested for three different concentrations i.e. 10 μg/ml, 20 μg/ml and 100 μg/ml. In general, isolates from root nodules showed higher resistance as compared to those of stem isolates. All the cultures showed resistance to neomycin and polymyxin B upto

Table 2. Antibiotic resistance spectra of *Azorhizobium* isolates

Azorhizobium isolates	Antibiotics µg/ml																				
	Kanamycin			Neomycin			Vancomycin			Erythromycin			Streptomycin			Polymyxin B			Nalidixic acid		
	10	**20**	**100**	**10**	**20**	**100**	**10**	**20**	**100**	**10**	**20**	**100**	**10**	**20**	**100**	**10**	**20**	**100**	**10**	**20**	**100**
SRS-1	+	+	-	+	+	+	-	-	-	+	+	-	+	+	-	+	+	-	-	-	-
SRS-3	-	-	-	+	+	-	-	-	-	+	+	-	+	+	-	+	+	-	-	-	-
SRS-7	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
SRS-10	+	-	-	+	+	-	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+
SRS-18	+	-	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	-	+	+	-
SRR-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
SRR-2	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	+
SRR-4	+	+	-	+	+	+	-	-	-	+	-	-	+	-	-	+	+	-	-	-	-
SRR-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SRR-6	+	-	-	+	+	+	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-
S1	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+
ZB-SK-5	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-

\+ Growth
\- No growth

Plate II. **Growth of *A. caulinodans* on YEMA supplemented with Bromothymol blue dye**

a. Control
b. Alkali producer
c. Acid producer

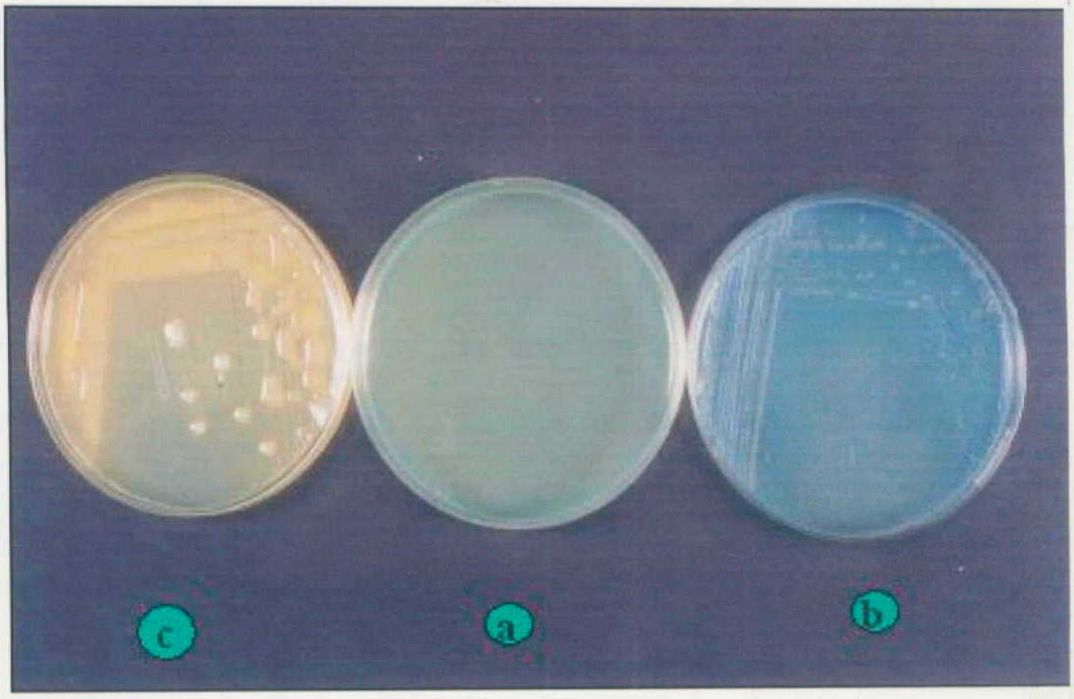

Plate II

concentration of 20 µg/ml. Root isolate SRR-1 and SRR-5, were found to be resistant to all antibiotics even upto concentration of 100 µg/ml, whereas other isolates showed variable resistance. Vancomycin was found to be inhibitory to most of the isolates. The least resistance was found in case of SRS-3 as it was sensitive to three antibiotics viz. kanamycin, vancomycin and nalidixic acid at all the three concentrations used.

The intrinsic antibiotic resistance spectra could be used for strain identification and for ecological studies. The intrinsic antibiotic resistance spectra of *Rhizobium* isolates were studied by Gupta *et al* (1983). The antibiotic resistance spectra of *A. caulinodans* isolates showed few similarities which could be due to the fact that they harbored almost same resistance plasmids.

4.1.5 Nitrogenase activity under free-living conditions

The *in vitro* nitrogenase activity of *A. caulinodans* isolates is presented in Table 3. The range of nitrogenase activity of isolates was recorded to be from 644.5-2718.6 nM C_2H_4 mg protein^{-1} h^{-1}. Among root isolate SRR-1 gave maximum acetylene reduction activity (2718.6 nM) followed by SRR-4 (1473.1 nM). While in stem isolates maximum activity was recorded by SRS-3 (2253.4 nM) followed by SRS-10 (1890.5 nM). The reference strains S_1 and *A. caulinodans* ZB-SK-5 gave 2229.6 nM and 883.9 nM C_2H_4 mg protein^{-1} h^{-1}, respectively. Dreyfus *et al* (1988) reported that root and stem nodulating strain could grow on N

Table 3. Nitrogenase activity of various *Azorhizobium* isolates under free living conditions

Azorhizobium isolates	Nitrogenase activity (nM C_2H_4 h^{-1} mg $protein^{-1}$)
SRS-1	1718.66
SRS-3	2253.36
SRS-7	1691.17
SRS-10	1890.53
SRS-18	1317.64
SRR-1	2718.62
SRR-2	1189.85
SRR-4	1473.14
SRR-5	644.50
SRR-6	1031.20
S1	2229.60
ZB-SK-5	883.88

under microaerobic conditions. Gebhardt *et al* (1984) reported nitrogenase activity upto 2000 nM C_2H_4 mg protein^{-1} h^{-1} under <9 μM dissolved oxygen continuous conditions. The root isolates thus recorded low nitrogenase activity as compared to stem isolates. The difference in nitrogen fixing capability could be due to the presence of intrinsic genetic variabilities and acquired ecological factors.

4.2 The effect of *Azorhizobium caulinodans* strains and phytohormones on rice cv. Bas-386 and cv. PR-116 under laboratory conditions

4.2.1 Root length

Bas-386 : Azorhizobial inoculation enhanced root length as compared to uninoculated control. S_1 gave maximum root length (15.3 cm) followed by ZB-SK-5 (Table 4). The phytohormone treatment alone decreased the root length. However, the dual treatment of *A. caulinodans* strains and phytohormones nullified the adverse effect of kinetin and GA_3 treatment alone (Plate III). The overall root length ranged from 9.9-11.8 cm and 7.6-10.1 cm with kinetin and GA_3 treatments respectively. Amongst the two phytohormones, kinetin performed better. Thus, the inoculation of *A. caulinodans* cultures resulted in subduing the effect of phytohormones.

PR-116 : The same trend was observed in rice seedlings cv. PR-116 as cv. Bas-386. Root length was found to be comparatively lesser in cv.

Table 4. Effect of inoculation of *Azorhizobium caulinodans* strains and phytohormones* on root length (cm) of rice seedlings 25 days after germination under laboratory conditions

Strain	Root length					
	var.Bas-386			var. PR-116		
	No Phytohormone	Kinetin	GA_3	No Phytohormone	Kinetin	GA_3
Control	9.94±0.83	7.50±0.50	7.00±0.74	6.51±0.53	5.38±0.50	5.00±0.68
SRR-1	12.50±0.94	9.90±0.85	10.08±0.63	8.82±0.84	7.65±1.25	7.00±0.57
S_1	15.25±0.55	11.80±0.52	8.62±0.49	7.88±0.48	7.44±0.52	6.84±0.83
ZB-SK-5	13.00±0.67	10.67±0.33	7.59±0.81	7.24±0.62	6.89±0.49	6.50±0.49

* Kinetin - 1.5 ppm
* Gibberellic acid - 3.0 ppm

Plate III. Effect of inoculation of *A. caulinodans* (SRR-1) and kinetin on root length of rice seedlings (cv. Bas-386)

a. Control
b. Kinetin alone
c. SRR-1 alone
d. Kinetin + SRR-1

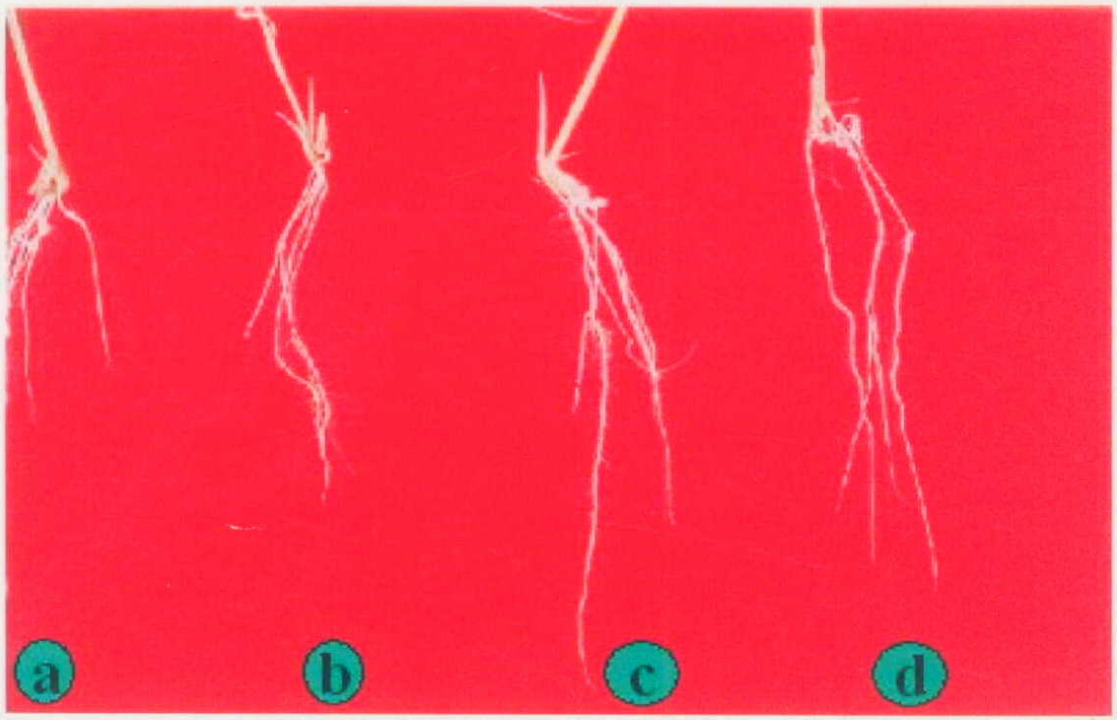

Plate III

PR-116 than cv. Bas-386. Root isolate SRR-1 recorded maximum root length (8.8 cm) followed by S_1 (Table 4).

4.2.2 Shoot length

Bas-386 : Inoculation of *Azorhizobium* strains alone increased shoot length that ranged from 19.2-22.1 cm as compared to uninoculated control (Table 5). Root isolate SRR-1 recorded 26% increase followed by S_1. On the other hand, treatment with phytohormones alone decreased the shoot length. However, the inoculation of *Azorhizobium* strains along with phytohormones compensated the decrease in shoot length. Kinetin proved better over GA_3.

PR-116 : In case of cv. PR-116 the shoot length was observed comparatively lesser than cv. Bas-386. Rice seedlings of cv. PR-116 also showed positive response to azorhizobial strain inoculation with maximum shoot length (17.5 cm) recorded by SRR-1 (Table 5). Kinetin performed better over GA_3 except when GA_3 was treated along with culture S_1.

4.2.3 Fresh weight

Bas-386 : An increase in fresh weight was observed in culture inoculated rice seedlings over the uninoculated control. SRR-1 treatment alone showed 28.5% increase in fresh weight followed by 19% increase by S_1 and ZB-SK-5 (Table 6). While, the phytohormone treatment alone showed decrease in fresh weight of plant. However, the inoculation of

Table 5. Effect of inoculation of *Azorhizobium caulinodans* strains and phytohormones* on shoot length (cm) of rice seedlings 25 days after germination under laboratory conditions

Strain	Shoot Length					
	var. Bas-386			var.PR-116		
	No Phytohormone	Kinetin	GA_3	No Phytohormone	Kinetin	GA_3
Control	17.55±0.64	15.63±0.64	15.01±0.81	14.00±0.67	10.00±0.77	8.98±0.50
SRR-1	22.11±0.67	21.66±1.78	20.37±1.05	17.50±0.89	15.25±0.84	13.99±0.47
S_1	20.85±1.31	18.98±0.46	18.40±0.69	15.68±1.50	11.30±0.79	12.08±0.66
ZB-SK-5	19.22±0.47	18.00±1.08	17.00±0.58	16.18±1.01	12.80±0.47	10.88±0.67

* Kinetin - 1.5 ppm
* Gibberellic acid - 3.0 ppm

Table 6. Effect of inoculation of *Azorhizobium caulinodans* strains and phytohormones* on fresh weight (g pl^{-1}) of rice seedlings 25 days after germination under laboratory conditions

Strain	Fresh weight					
	var. Bas-386			var.PR-116		
	No Phytohormone	Kinetin	GA_3	No Phytohormone	Kinetin	GA_3
Control	0.042±0.005	0.036±0.003	0.035±0.004	0.045±0.005	0.038±0.004	0.035±0.003
SRR-1	0.054±0.003	0.052±0.006	0.045±0.005	0.065±0.005	0.057±0.003	0.051±0.005
S_1	0.050±0.006	0.040±0.005	0.045±0.005	0.058±0.004	0.046±0.005	0.050±0.005
ZB-SK-5	0.050±0.005	0.045±0.005	0.040±0.004	0.051±0.004	0.048±0.005	0.046±0.004

* Kinetin - 1.5 ppm
* Gibberellic acid - 3.0 ppm

A. caulinodans strains along with phytohormones suppressed the negative effect of phytohormones when treated alone. Kinetin proved better over GA_3 except when GA_3 was treated along with culture S_1.

PR-116 : Rice seedlings of cv. PR-116 followed the same trend as cv. Bas-386. Root isolate SRR-1 showed 50.0% increase followed by 26.3% increase by ZB-SK-5. The overall fresh weight ranged from 0.046-0.057 g and 0.046-0.051 g in kinetin and GA_3 treatment respectively (Table 6). The plant fresh weight was found to be comparatively more in cv. PR-116 than cv. Bas-386.

4.2.4 Dry weight

Bas-386 : *A. caulinodans* strains inoculated rice seedlings enhanced the dry weight over the uninoculated control. Among the culture inoculated seedlings, SRR-1 gave maximum dry weight (5.8 mg) followed by S_1 (Table 7). On the other hand, phytohormone treatment alone reduced the dry weight over untreated control. However, the dual treatment of azorhizobial cultures along with phytohormones compensated the decrease in dry weight. Among the two phytohormones, kinetin gave better results over GA_3 except when GA_3 was treated in combination with S_1.

PR-116 : The dry weight was observed comparatively more in cv. PR-116 than cv. Bas-386. SRR-1 produced maximum dry weight (6.9 mg)

Table 7. Effect of inoculation of *Azorhizobium caulinodans* strains and phytohormones* on dry weight (mg pl^{-1}) of rice seedlings 25 days after germination under laboratory conditions

Strain	Dry weight					
	var.Bas-386			var.PR-116		
	No Phytohormone	Kinetin	GA_3	No Phytohormone	Kinetin	GA_3
Control	4.80±0.42	3.70±0.50	3.55±0.68	4.18±0.68	3.50±0.42	3.19±0.72
SRR-1	5.80±0.58	4.65±0.89	4.85±0.78	6.94±0.26	5.50±0.25	4.85±0.71
S_1	5.25±0.77	4.25±0.95	4.70±0.65	5.84±0.78	4.80±0.58	5.00±0.59
ZB-SK-5	4.85±0.67	4.10±0.30	4.00±0.49	5.15±0.52	4.50±0.37	4.34±0.84

* Kinetin - 1.5 ppm
* Gibberellic acid - 3.0 ppm

followed by S_1 (Table 7). However, both the varieties followed the similar trends.

The decrease in root-shoot length and biomass by phytohormone alone treatment may be due to increased cell division without undergoing cell elongation and maturation stages resulting in shortened root system which becomes unable to absorb more nutrients and thus a consequent decrease in shoot length as well as biomass.

The subduing of deleterious effects of phytohormone alone treatment could be reversed to an extent by inoculation of *A. caulinodans* and this may be attributed to catabolization of phytohormones by bacteria, thus decreasing the concentration of the applied phytohormone around root surface or may be due to unknown factor that may be produced only in presence of bacteria or by host plant that neutralizes excess amount of applied phytohormone.

The increase in all growth characters by *A. caulinodans* alone treatment may be due to PGPR like action of this bacteria or may be due to secretion of phytohormone in the vicinity of roots.

The present findings agree with the results obtained by Kannaiyan *et al* (2001) reporting that the inoculation of *A. caulinodans* strains increased root and shoot growth, total biomass and lateral rootlets of rice seedlings over the uninoculated control.

They also reported that the inoculation of *A. caulinodans* could nullify the adverse effect of kinetin to some extent compared to the incorporation of growth regulator treatment alone. Similarly, Buvana and Kanaiyan (1998) also reported that 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.

4.2.5 Paranodule number

Bas-386 : No paranodules were observed in the uninoculated control while inoculation of SRR-1 and S_1 alone showed formation of very less number of paranodules. Similarly, the phytohormone treatment alone produced no paranodules. Whereas, the number of paranodules enhanced with the inoculation of *A. caulinodans* strains in combination with the phytohormones. Among the various treatments, combination of kinetin with SRR-1 gave maximum number of paranodules (19.0) (Plate IV) followed by S_1 (Table 8). Kinetin worked better than GA_3.

PR-116 : Paranodules were found to be absent when *A. caulinodans* strains and phytohormones were treated separately except inoculation of SRR-1 alone. The dual treatment of S_1 along with kinetin produced maximum number of paranodules (28.0) (Plate V) followed by SRR-1. The number of paranodules were relatively more in rice seedlings of cv. PR-116 than cv. Bas-386 (Table 8).

Table 8. Effect of inoculation of *Azorhizobium caulinodans* strains and phytohormones* on paranodule formation of rice seedlings 25 days after germination under laboratory conditions

Strain	Paranodule number per plant					
	var. Bas-386			var.PR-116		
	No Phytohormone	Kinetin	GA_3	No Phytohormone	Kinetin	GA_3
Control	-	-	-	-	-	-
SRR-1	3.42±0.57	19.00±0.68	14.91±0.54	3.00±0.55	24.00±0.71	16.88±0.80
S_1	2.31±0.54	16.87±0.95	13.91±0.84	-	28.00±0.55	15.00±1.06
ZB-SK-5	-	12.91±0.32	10.67±0.54	-	22.67±0.98	17.90±1.02

* Kinetin - 1.5 ppm
* Gibberellic acid - 3.0 ppm

Plate IV. **Paranodules on roots of rice cv. Bas-386 in presence of kinetin and *A. caulinodans* (40X x 10X)**

Plate V. **Paranodules on roots of rice cv. PR-116 in presence of kinetin and *A. caulinodans* (40X x 10X)**

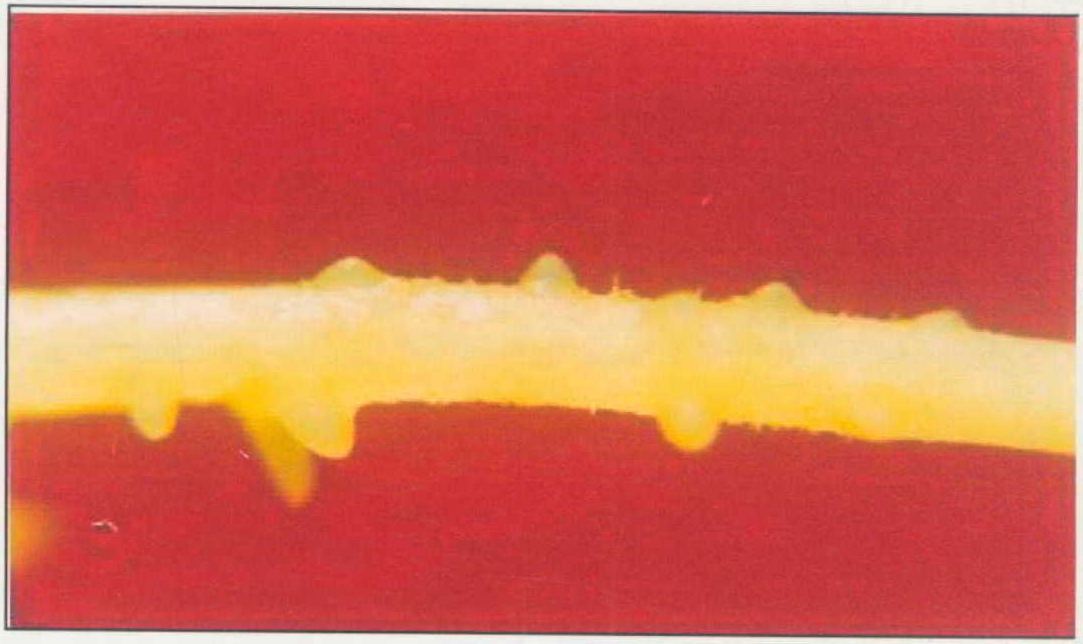

Plate IV

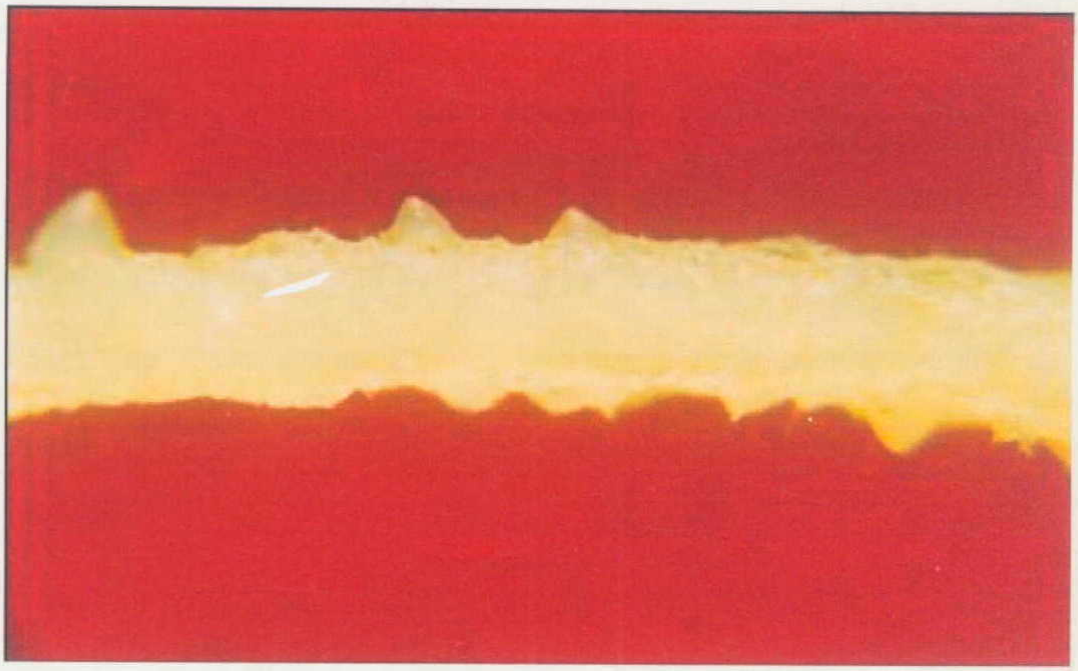

Plate V

4.2.6 Lateral rootlet number

Bas-386 : The treatment of *A. caulinodans* strains and phytohormones separately increased the number of lateral rootlets formed per plant over the untreated control. SRR-1 formed maximum number of lateral rootlets (91.4) followed by ZB-SK-5 (Table 9). The inoculation of *A. caulinodans* strains along with kinetin showed comparable results with that of kinetin treatment alone. The overall lateral rootlet number ranged from 73.9 to 80.9 and 65.3 to 78.7 in kinetin and GA_3 treatment respectively. Kinetin worked better than GA_3.

PR-116 : The number of lateral rootlets were comparatively more in cv. PR-116 than cv. Bas-386 (Table 9). The similar trend was followed in both the varieties. Maximum number of lateral rootlets were observed by SRR-1 (118.0) followed by S_1.

4.2.7 Nitrogenase activity

Bas-386 : No nitrogenase activity was observed in uninoculated as well as phytohormone treated control. Likewise nitrogenase activity was not observed in culture inoculated rice seedlings except SRR-1 which recorded very low nitrogenase activity. The nitrogenase activity enhanced tremendously when *A. caulinodans* strains were inoculated along with phytohormones. Kinetin and GA_3 gave maximum nitrogenase activity in combination with SRR-1 and ZB-SK-5 respectively (Table 10).

Table 9. Effect of inoculation of *Azorhizobium caulinodans* strains and phytohormones* on lateral root formation of rice seedlings 25 days after germination under laboratory conditions

Strain	Lateral rootlets per plant					
	var. Bas-386			var.PR-116		
	No Phytohormone	Kinetin	GA_3	No Phytohormone	Kinetin	GA_3
Control	62.7±0.35	75.2±0.46	70.91±0.78	76.50±0.90	85.30±0.78	80.00±1.45
SRR-1	91.42±0.29	79.40±0.58	65.33±0.99	118.00±0.86	91.95±0.50	68.56±0.53
S_1	86.95±0.53	73.98±0.66	70.80±0.47	105.01±0.88	85.96±0.64	80.90±0.95
ZB-SK-5	89.54±1.42	80.88±1.26	78.67±0.67	95.50±0.36	80.00±0.55	75.56±0.71

* Kinetin - 1.5 ppm
* Gibberellic acid - 3.0 ppm

Table 10. Effect of inoculation of *Azorhizobium caulinodans* strains and phytohormones* on nitrogenase activity ($\mu M\ pl^{-1}\ 24h^{-1}$) of rice seedlings 25 days after germination under laboratory conditions

Strain	Nitrogenase activity					
	var. Bas-386			var.PR-116		
	No Phytohormone	Kinetin	GA_3	No Phytohormone	Kinetin	GA_3
Control	-	-	-	-	-	-
SRR-1	2.50± 0.19	13.50±0.38	9.82±0.54	2.00±0.16	16.98± 0.62	12.85±0.38
S1	-	12.70±0.92	9.33±0.75	-	13.40 ±0.47	12.00±0.69
ZB-SK-5	-	13.00±0.49	10.54±0.81	-	15.00 ±0.98	12.46±0.43

* Kinetin - 1.5 ppm
* Gibberellic acid - 3.0 ppm

PR-116 : The same trend was observed in rice seedlings cv. PR-116 as cv. Bas-386. The overall nitrogenase activity ranged from 13.4-16.9 μM and 12.0-12.8 μM C_2H_4 pl^{-1} 24 h^{-1} in kinetin and GA_3 treatments respectively with SRR-1 showing maximum activity in both cases (Table 10). The nitrogenase activity was recorded relatively more in cv. PR-116 than cv. Bas-386.

The phytohormone treatment increased the number of lateral rootlets on account of their effect on pericycle or stele. Nodule like structures are also the modified lateral rootlets. In presence of *A. caulinodans* strains, phytohormones enhanced the crack entry and thus increased the number of modified lateral rootlets i.e. paranodule formation. Due to increased paranodule number and *A. caulinodans* infections, nitrogenase activity could be observed by ARA.

The present results are in line with those of Amutha and Kannaiyan (1998) who found the stimulation of lateral root 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 (1999) also reported increased nodulation and nitrogenase activity by *A. caulinodans* inoculation along with 2,4-D and kinetin treatment in rice, cumbu, ragi and maize seedlings.

Amutha and Kannaiyan (2000) reported absence of nodule like structures in rice roots when *A. caulinodans* and growth regulator kinetin

were treated in the seedlings separately. Kannaiyan *et al* (2001) also reported that increased concentration of kinetin with inoculation of *A. caulinodans* increased the number of nodule like structures in the rice roots.

4.2.8 Competitiveness of inoculated strains

The per cent establishment of azorhizobial strains was observed to be 100%. Both the reference strains S1 and ZB-SK-5 as well as the new isolate SRR-1 showed presence in all the inoculated rice plant roots under laboratory conditions. The YEMA medium containing antibiotic concentrations to which the respective azorhizobial strains were resistant gave positive results on streaking with the extract taken from surface sterilized roots of rice seedlings. This shows the movement of inoculated bacteria from exterior of roots to get established internally in cortex (outer) or endophytically in roots.

4.3 The effect of *Azorhizobium caulinodans* strains, 2,4-D and N application on yield and yield attributing characters of cv. Bas-386 and cv. PR-116 under field conditions

4.3.1 Root length

Bas-386 : N-levels and 2,4-D application did not show any significant response in the root length of the plants at all stages of sampling, however at 30DAT the root length responded significantly to the 2,4-D treatment. All azorhizobial strains performed significantly better over the control at all stages of sampling. At 15DAT all the

azorhizobial strains performed statistically at par amongst themselves. While at 30DAT and at harvesting time, the maximum root length was produced by root isolate SRR-1 and both the reference cultures were found to be statistically at par amongst themselves (Table 11).

PR-116 : At 15DAT, the result of the experiment did not show any significant increase with the application of all the strains of *Azorhizobium* or by use of 2,4-D and N-fertilizer application. However, 30DAT root length was found to be significantly increased with azorhizobial inoculation and by use of N-fertilizer. The mean value of ¾ N and full N were found to be statistically at par amongst themselves. Whereas 2,4-D treatment showed significant increase at the time of harvesting. The root length responded significantly to all the strains of *Azorhizobium*. Both the reference strains performed significantly better over the control (Table 12).

4.3.2 Shoot length

Bas-386 : The result of the treatment did not show any significant response in the shoot length with the application of 2,4-D during every sampling. However, 15DAT and at harvesting time significant difference was observed between both the N-levels. At 30DAT and at harvesting stage, all azorhizobial strains performed significantly better over the control. The cultures performed statistically at par amongst themselves (Table 13).

Table 11 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on root length (cm) of rice cv. Bas-386 under field conditions

11(a) 15 DAT

Strains (C)	Root Length				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	8.56	10.16	10.26	12.16	10.29
SRR-1	13.33	18.50	13.66	13.50	14.75
S1	11.83	15.83	13.66	16.00	14.33
ZB-SK-5	15.83	11.83	14.33	17.60	14.92
Mean(N)	13.24		13.91		
Mean(2,4-D)	12.68(0.0ppm)		14.46(3.0ppm)		

CD(5%)** **C**-2.62

11(b) 30 DAT

Control	17.33	17.00	18.66	18.16	17.79
SRR-1	23.83	25.50	19.33	26.33	23.75
S1	25.50	22.30	18.00	23.50	22.33
ZB-SK-5	19.50	26.83	22.00	21.16	22.37
Mean(N)	22.33		20.89		
Mean(2,4-D)	20.52		22.60		

CD(5%)** **B**-2.03 **C**-2.87 **ABC**-5.73

11(c)At the time of harvesting

Control	17.00	18.50	17.33	17.66	17.63
SRR-1	25.33	28.33	22.50	27.33	25.87
S1	22.66	21.83	20.83	21.83	21.79
ZB-SK-5	21.66	21.00	22.00	21.33	21.13
Mean(N)	21.85		21.35		
Mean(2,4-D)	20.98		22.23		

CD(5%)** **C**-1.83

** CD (5%) for all other treatments/interactions is NS

Table 12 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on root length (cm) of rice cv. PR-116 under field conditions

12(a) 15 DAT

Strains (C)	Root length				Mean
	N-levels (A)				
	¾ N		N (Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	17.00	14.83	15.66	16.17	15.92
SRR-1	19.00	23.00	15.00	21.00	19.50
S1	18.17	18.16	18.50	19.50	18.58
ZB-SK-5	17.66	16.83	19.66	21.50	18.92
Mean(N)	18.08		18.37		
Mean(2,4-D)	17.58 (0.0 ppm)		18.87(3.0 ppm)		

CD (5%)**

12(b) 30 DAT

Control	15.33	16.00	19.00	20.16	17.63
SRR-1	25.00	25.00	24.00	31.16	26.29
S1	21.33	19.33	24.00	20.50	21.29
ZB-SK-5	20.66	20.50	21.66	19.33	20.42
Mean(N)	20.39		22.42		
Mean(2,4-D)	21.31		21.50		

CD (5%)** **A**-1.98 **C**-2.80

12(c)At the time of harvesting

Control	14.16	16.50	13.00	18.50	15.54
SRR-1	19.66	21.33	21.83	21.50	21.08
S1	20.50	17.83	17.83	20.50	19.16
ZB-SK-5	18.50	19.83	16.66	23.66	19.66
Mean(N)	18.54		19.19		
Mean(2,4-D)	17.77		19.95		

CD (5%)** **B**-1.81 **C**-2.55

** CD (5%) for all other treatments/interactions is NS

Table 13 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on shoot length (cm) of rice cv. Bas-386 under field conditions

13(a) 15 DAT

Strains (C)	Shoot Length				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	33.83	36.83	28.66	31.00	32.58
SRR-1	36.66	48.33	39.33	39.33	40.92
S1	46.33	42.66	34.66	39.00	40.66
ZB-SK-5	42.33	41.00	40.33	41.00	41.16
Mean(N)	41.00		36.66		
Mean(2,4-D)	37.77(0.0ppm)		39.89(3.0ppm)		

CD(5%)** **A**-2.15 **B**-3.04 **ABC**-6.08

13(b) 30 DAT

Control	54.33	57.33	59.66	58.33	57.42
SRR-1	75.00	86.00	67.00	70.66	74.66
S1	69.66	72.66	69.66	69.00	70.25
ZB-SK-5	78.00	67.00	70.66	71.66	71.83
Mean(N)	70.00		67.08		
Mean(2,4-D)	68.00		69.08		

CD(5%)** **C**-5.15 **AC**-7.93

13(c)At the time of harvesting

Control	93.33	104.33	101.66	99.33	99.66
SRR-1	140.00	155.00	131.66	116.66	135.83
S1	126.66	127.33	111.00	119.33	121.08
ZB-SK-5	129.66	145.33	125.00	122.66	130.66
Mean(N)	127.71		115.92		
Mean(2,4-D)	119.87		123.75		

CD(5%)** **A**-9.81 **C**-13.87

** CD (5%) for all other treatments/interactions is NS

PR-116 : Application of N or 2,4-D did not show any response to shoot length at all stages of growth. However, on an average all the *Azorhizobium* strains gave significantly higher values over the control (Table 14).

4.3.3 Root fresh weight

Bas-386 : The results showed a significant increase in the root fresh weight between both the N levels upto 30 DAT. However, 2,4-D significantly enhanced root fresh weight at harvesting time. Significant increase in the root fresh weight was also observed with application of all strains of *Azorhizobium* over the control at all stages of sampling (Table 15).

PR-116 : The significant increase in root fresh weight was observed at 15DAT with 2,4-D application whereas at harvesting time with N-application. All azorhizobial strains performed significantly better over the control at all stages of sampling and also found to be statistically at par amongst themselves (Table 16).

4.3.4 Shoot fresh weight

Bas-386 : The result showed significant increase in shoot fresh weight at 15DAT with N application whereas at 30DAT and at harvesting time with 2,4-D application. All azorhizobial isolates worked significantly better over the control at all stages of sampling. The best performer was

Table 14 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on shoot length (cm) of rice cv. PR-116 under field conditions

14(a) 15 DAT

Strains (C)	Shoot Length				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	34.16	36.00	37.00	30.50	34.42
SRR-1	50.66	57.83	50.66	50.00	52.54
S1	49.50	47.16	49.50	51.50	49.87
ZB-SK-5	47.00	51.66	47.00	48.83	49.63
Mean(N)	46.69		46.54		
Mean(2,4-D)	46.60(0.0ppm)		46.63(3.0ppm)		

CD (5%)** **AB**-3.69 **C**-3.69

14(b) 30 DAT

Control	65.33	62.66	63.66	61.50	63.29
SRR-1	86.9	86.16	83.83	91.33	87.06
S1	88.00	78.83	77.83	80.00	81.16
ZB-SK-5	79.33	85.00	82.50	84.66	82.87
Mean(N)	79.029		78.166		
Mean(2,4-D)	78.43		78.77		

CD (5%)** **C**-3.66

14(c) At the time of harvesting

Control	78.00	81.33	80.00	73.33	78.16
SRR-1	104.00	100.33	112.66	102.66	104.92
S1	109.00	108.33	100.66	94.00	103.00
ZB-SK-5	98.66	94.33	109.00	98.00	100.00
Mean(N)	99.00		94.04		
Mean(2,4-D)	96.75		96.29		

CD(5%)** **C**-7.15 **BC**-10.12

** CD (5%) for all other treatments/interactions is NS

Table 15 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on root fresh weight (g pl^{-1}) of rice cv. Bas-386 under field conditions

15(a) 15 DAT

Strains (C)	Root fresh weight				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	0.32	0.85	0.53	0.42	0.53
SRR-1	1.15	1.62	1.04	0.82	1.16
S1	0.90	1.18	0.55	0.96	0.90
ZB-SK-5	1.22	0.78	0.78	0.96	0.93
Mean(N)	1.00		0.75		
Mean(2,4-D)	0.81(0.0ppm)		0.94(3.0ppm)		

CD(5%)** **A**-0.15 **C**-0.21 **ABC**-0.41

15(b) 30 DAT

Control	2.54	3.15	2.69	2.45	2.71
SRR-1	5.86	4.95	4.67	4.33	4.95
S1	7.76	5.61	4.22	4.95	5.63
ZB-SK-5	5.80	5.79	4.36	3.73	4.92
Mean(N)	5.18		3.93		
Mean(2,4-D)	4.74		4.37		

CD(5%)** **A**-0.83 **C**-1.17

15(c)At the time of harvesting

Control	6.11	6.95	5.62	7.22	6.47
SRR-1	12.13	16.97	13.74	17.39	15.05
S1	13.04	15.80	15.68	17.38	15.48
ZB-SK-5	11.83	13.65	12.06	14.97	13.13
Mean(N)	12.06		13.00		
Mean(2,4-D)	11.27		13.79		

CD(5%)** **B**-1.51 **C**-2.13

** CD (5%) for all other treatments/interactions is NS

Table 16 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on root fresh weight (g pl^{-1}) of rice cv. PR-116 under field conditions

16(a) 15 DAT

Strains (C)	Root fresh weight				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	2.04	2.10	1.92	2.51	2.14
SRR-1	4.14	5.27	4.55	5.75	4.93
S1	4.23	4.66	3.29	4.12	4.08
ZB-SK-5	2.78	5.83	3.83	3.92	4.09
Mean(N)	3.88		3.73		
Mean(2,4-D)	3.34(0.0ppm)		4.27(3.0ppm)		

CD(5%)** **B**-0.47 **C**-0.67 **ABC**-1.34

16(b) 30 DAT

Control	9.84	10.02	10.25	13.06	10.79
SRR-1	12.23	19.48	20.64	17.63	17.49
S1	17.04	12.85	19.39	14.16	15.86
ZB-SK-5	16.85	18.79	14.71	18.62	17.24
Mean(N)	14.64		16.06		
Mean(2,4-D)	15.12		15.57		

CD(5%)** **C**-2.99

16(c)At the time of harvesting

Control	16.34	19.77	16.81	12.95	16.47
SRR-1	35.86	26.63	29.88	25.92	29.57
S1	28.65	24.47	27.87	24.68	26.42
ZB-SK-5	24.27	21.35	42.75	30.85	29.80
Mean(N)	27.81		23.33		
Mean(2,4-D)	24.67		26.46		

CD(5%)** **A**-3.01 **C**-4.26

** CD (5%) for all other treatments/interactions is NS

isolate SRR-1 while both the reference strains were found to be at par amongst themselves (Table 17).

PR-116 : The significant difference was observed between both the N-levels upto 30DAT. The ¾ N level worked significantly better over the full N level. However, the 2,4-D application significantly enhanced the shoot fresh weight at 30DAT. Significant increase was observed with *Azorhizobium* strains over the control at all stages of sampling. The best performer was root isolate SRR-1 and both the reference strains were found to be statistically at par amongst themselves (Table 18).

4.3.5 Root dry weight

Bas-386 : The result of the experiment did not show any significant response in the root dry weight with application of all treatments upto 15DAT. However, at 30DAT and at harvesting time, the significant difference was observed between both the N-levels whereas for 2,4-D levels at 30DAT. The root dry weight showed significant increase with all *Azorhizobium* strains. Maximum root dry weight was produced by SRR-1 (Table 19).

PR-116 : The root dry weight enhanced significantly with 2,4-D application at 15DAT and at harvesting time whereas with N application at 30 DAT and at harvesting time. All the cultures performed statistically better over control at all stages of growth with SRR-1 as best performer upto 30DAT while S_1 at harvesting time (Table 20).

Table 17 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on shoot fresh weight (g pl^{-1}) of rice cv. Bas-386 under field conditions

17(a) 15 DAT

Strains (C)	Shoot fresh weight				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	0.76	1.36	1.02	1.12	1.07
SRR-1	2.79	3.66	1.54	1.21	2.30
S1	2.64	2.03	1.66	2.45	2.19
ZB-SK-5	2.38	1.75	1.24	1.56	1.73
Mean(N)	2.17		1.47		
Mean(2,4-D)	1.75(0.0ppm)		1.89(3.0ppm)		

CD(5%)** **A**-0.36 **C**-0.51 **AC**-0.73

17(b) 30 DAT

Control	8.96	10.28	9.21	10.06	9.63
SRR-1	16.65	19.52	13.17	16.54	16.48
S1	16.24	18.64	8.52	14.87	14.57
ZB-SK-5	11.49	17.65	14.07	15.35	14.64
Mean(N)	14.93		12.73		
Mean(2,4-D)	12.29		15.37		

CD(5%)** **B**-2.44 **C**-3.44

17(c)At the time of harvesting

Control	15.11	16.88	17.16	16.30	16.36
SRR-1	23.15	26.45	24.39	27.03	25.26
S1	22.13	25.44	21.95	24.08	23.40
ZB-SK-5	19.03	21.41	19.43	21.49	20.34
Mean(N)	21.20		21.48		
Mean(2,4-D)	20.29		22.39		

CD(5%)** **B**-1.58 **C**-2.24

** CD (5%) for all other treatments/interactions is NS

Table 18 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on shoot fresh weight (g pl^{-1}) of rice cv. PR-116 under field conditions

18(a) 15 DAT

Strains (C)	Shoot fresh weight				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	7.22	8.98	8.44	8.82	8.37
SRR-1	13.53	11.67	9.86	12.12	11.79
S1	11.14	10.73	9.84	10.17	10.47
ZB-SK-5	8.82	14.62	7.83	10.10	10.34
Mean(N)	10.84		9.65		
Mean(2,4-D)	9.58(0.0ppm)		10.90(3.0ppm)		

CD(5%)** **A**-0.86 **B**-0.86 **C**-1.22

18(b) 30 DAT

Control	29.34	26.99	33.63	30.13	30.02
SRR-1	55.37	57.20	44.99	44.08	50.41
S1	47.34	49.08	35.23	30.96	40.65
ZB-SK-5	48.69	48.70	35.72	42.95	44.02
Mean(N)	45.34		37.21		
Mean(2,4-D)	41.29		41.26		

CD(5%)** **A**-5.45 **C**-7.70

18(c)At the time of harvesting

Control	73.33	60.00	90.00	86.66	77.50
SRR-1	146.66	170.00	111.66	130.00	139.58
S1	106.66	150.00	121.66	80.00	114.58
ZB-SK-5	98.33	148.33	133.33	113.33	123.33
Mean(N)	119.16		108.33		
Mean(2,4-D)	110.21		117.29		

CD(5%)** **AB**-18.25 **C**-18.25 **AC**-25.81 **ABC**-36.50

** CD (5%) for all other treatments/interactions is NS

Table 19 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on root dry weight (g pl^{-1}) of rice cv. Bas-386 under field conditions

19(a) 15 DAT

Strains (C)	Root dry weight				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	0.12	0.15	0.12	0.12	0.13
SRR-1	0.14	0.16	0.15	0.18	0.16
S1	0.16	0.14	0.10	0.13	0.133
ZB-SK-5	0.21	0.16	0.12	0.16	0.16
Mean(N)	0.15		0.13		
Mean(2,4-D)	0.14(0.0ppm)		0.14(3.0ppm)		

CD(5%)**

19(b) 30 DAT

Control	1.46	1.93	1.56	1.95	1.73
SRR-1	3.93	3.88	4.46	4.30	4.14
S1	2.83	3.26	2.90	3.39	3.09
ZB-SK-5	2.43	3.90	3.00	4.00	3.33
Mean(N)	2.95		3.19		
Mean(2,4-D)	2.82		3.33		

CD(5%)** **A**-0.18 **B**-0.18 **C**-0.25 **BC**-0.36

19(c)At the time of harvesting

Control	4.24	4.44	4.62	4.01	4.33
SRR-1	6.25	6.78	6.77	7.09	6.72
S1	5.09	5.53	6.14	5.84	5.65
ZB-SK-5	4.46	5.09	5.26	4.95	4.94
Mean(N)	5.24		5.59		
Mean(2,4-D)	5.35		5.47		

CD(5%)** **A**-0.24 **AB**-0.34 **C**-0.34

** CD (5%) for all other treatments/interactions is NS

Table 20 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on root dry weight (g pl^{-1}) of rice cv. PR-116 under field conditions

20(a) 15 DAT

Strains (C)	Root dry weight				Mean
	N-levels (A)				
	¾ N		N (Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	1.33	1.76	1.27	1.68	1.51
SRR-1	1.88	2.82	1.90	2.77	2.34
S1	2.28	2.41	2.24	2.20	2.28
ZB-SK-5	1.68	2.06	1.82	2.11	1.92
Mean(N)	2.03		2.00		
Mean(2,4-D)	1.80(0.0ppm)		2.23(3.0ppm)		

CD(5%)** **B**-0.18 **C**-0.25 **BC**-0.36

20(b) 30 DAT

Control	3.14	3.24	2.57	3.98	3.23
SRR-1	6.24	8.40	4.88	4.88	6.10
S1	6.84	3.85	5.04	4.99	5.18
ZB-SK-5	4.56	4.02	3.96	3.32	3.97
Mean(N)	5.04		4.20		
Mean(2,4-D)	4.66		4.58		

CD(5%)** **A**-0.59 **C**-0.83 **ABC**-1.66

20(c)At the time of harvesting

Control	8.48	10.09	10.44	10.33	9.90
SRR-1	13.48	18.85	14.89	19.99	16.80
S1	18.83	19.00	18.20	19.14	18.79
ZB-SK-5	10.92	12.29	11.36	12.85	11.86
Mean(N)	14.03		14.65		
Mean(2,4-D)	13.36		15.32		

CD(5%)** **A**-0.60 **B**-0.60 **C**-0.85 **BC**-1.20

** CD (5%) for all other treatments/interactions is NS

4.3.6 Shoot dry weight

Bas-386 : The significant difference was observed among the plants between both the N-levels at the time of harvesting. However, 2,4-D application enhanced shoot dry weight at 30DAT and at harvesting time. All the azorhizobial isolates performed significantly better over the control at all the stages of sampling. All the cultures were found to be statistically at par amongst themselves at 15 DAT while the root isolate SRR-1 recorded maximum shoot dry weight followed by S_1 (Table 21).

PR-116 : No significant difference was observed between both the N-levels at all the stages of sampling. However, the root dry weight responded significantly to the 2,4-D treatment at 30DAT and at harvesting time. The best performer among all strains was SRR-1 followed by S_1. At 15DAT both the reference strains were found to be statistically at par with the control while at harvesting time all cultures performed significantly better over the control (Table 22).

Rhizobial inoculation enhanced the growth promoting activities (GPA) which can be attributed to production of phytohormones like IAA and GA in the external root environment, fungal growth inhibition, production and secretion of siderophores, more efficient use of N sources and other nutrients, increased N uptake, feeble N_2 fixation, antibiosis against phytopathogens. These GPA may regulate the growth promotion

Table 21 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on shoot dry weight (g pl^{-1}) of rice cv. Bas-386 under field conditions

21(a) 15 DAT

Strains (C)	Shoot dry weight				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	0.23	0.32	0.40	0.26	0.30
SRR-1	0.49	0.61	0.45	0.43	0.49
S1	0.53	0.45	0.41	0.40	0.45
ZB-SK-5	0.44	0.37	0.38	0.42	0.41
Mean(N)	0.43		0.39		
Mean(2,4-D)	0.42(0.0ppm)		0.41(3.0ppm)		

CD(5%)** **C**-0.07 **ABC**-0.14

21(b) 30 DAT

Control	2.52	2.86	2.53	2.77	2.67
SRR-1	4.73	5.23	4.87	5.16	4.99
S1	3.81	4.45	4.49	4.43	4.24
ZB-SK-5	3.40	4.02	3.25	4.01	3.67
Mean(N)	3.85		3.94		
Mean(2,4-D)	3.70		4.09		

CD(5%)** **B**-0.20 **C**-0.28

21(c)At the time of harvesting

Control	7.45	9.44	7.03	10.21	8.53
SRR-1	15.39	17.76	16.52	19.74	17.35
S1	14.29	14.72	16.52	17.04	15.65
ZB-SK-5	13.23	14.19	14.35	15.91	14.42
Mean(N)	13.31		14.66		
Mean(2,4-D)	13.09		14.88		

CD(5%)** **A**-1.02 **B**-1.02 **C**-1.44

** CD (5%) for all other treatments/interactions is NS

Table 22 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on shoot dry weight (g pl^{-1}) of rice cv. PR-116 under field conditions

22(a) 15 DAT

Strains (C)	Shoot dry weight				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	5.98	6.45	5.82	6.72	6.24
SRR-1	9.15	10.13	8.16	9.15	9.15
S1	7.86	6.86	7.19	7.05	7.24
ZB-SK-5	7.52	9.07	6.04	6.78	7.35
Mean(N)	7.88		7.11		
Mean(2,4-D)	7.21(0.0ppm)		7.77(3.0ppm)		

CD(5%)** **C**-1.18

22(b) 30 DAT

Control	9.69	10.48	9.65	11.64	10.37
SRR-1	15.27	18.18	15.42	17.67	16.64
S1	11.15	13.23	14.27	16.92	13.89
ZB-SK-5	12.55	12.79	12.04	13.78	12.79
Mean(N)	12.92		13.93		
Mean(2,4-D)	12.51		14.34		

CD(5%)** **B**-1.26 **C**-1.79

22(c)At the time of harvesting

Control	15.15	17.66	14.66	17.07	16.13
SRR-1	22.13	28.64	21.59	30.24	25.65
S1	20.57	21.15	22.07	21.31	21.28
ZB-SK-5	18.03	19.93	19.01	19.80	19.19
Mean(N)	20.41		20.72		
Mean(2,4-D)	19.15		21.98		

CD(5%)** **B**-1.31 **C**-1.85 **BC**-2.61

** CD (5%) for all other treatments/interactions is NS

in rice plant and thus may contribute to increase in growth characters viz. root-shoot length, root-shoot fresh and dry weight.

Chaintreuil *et al* (2000) reported 20% increase in shoot growth by inoculation with one endophytic strain ORS2011 and *Aeschynomene* photosynthetic *Bradyrhizobium* strain ORS278.

Biswas *et al* (2000a) reported the accumulation of IAA in external root environment of rice plants inoculated with rhizobia which further regulate the growth promotion in rice plants.

Zelitch (1982) observed that at least 90% of the biomass of higher plants is derived from CO_2 assimilated through photosynthesis. The increase in single leaf / or whole plant photosynthetic rate due to rhizobial inoculation as reported by Peng *et al* (2002) may attribute to increase in total biomass of rice plants.

Biswas *et al* (2000b) reported higher shoot dry matter (DM) accumulation which may be due to improved photosynthetic capacity or may be due to higher nutrient uptake efficiency.

4.3.7 Nitrogenase activity

Bas-386 : At all stages of sampling, the results showed significant increase in the root nitrogenase activity with application of 2,4-D and *Azorhizobium* strains. On the other hand, N application did not help to enhance acetylene reduction activity at all stages except at 30DAT. All the cultures performed significantly better over the control. The root

isolate SRR-1 worked significantly better over the control followed by S_1 (Table 23).

PR-116 : The significant increase in activity was observed with increasing dose of N at harvesting time. While, acetylene reduction activity enhanced significantly by 2,4-D application and all *Azorhizobium* strains also worked significantly better over the control. The maximum activity was recorded by SRR-1 followed by ZB-SK-5 (Table 24).

4.3.8 Paranodule number

Bas-386 : Paranodules were observed only at 15DAT (Plate VI) and their number was relatively more at low level of N application. A non significant increase was observed by 2,4-D application. All strains performed significantly better over the control. The best performer among all was recorded as SRR-1 which was also found to be statistically at par with reference strain S_1 (Table 25).

PR-116 : The mean values of ¾ N and full N level was found to be statistically at par amongst themselves at 15DAT. Whereas, the paranodule number enhanced significantly by application of 2,4-D and *Azorhizobium* strains also worked significantly better over the control (Plate VII). Maximum number of paranodules were recorded by SRR-1 while both the reference strains were found to be statistically at par amongst themselves. However, paranodules were found to be absent at harvesting time (Table 26).

Table 23 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on root nitrogenase activity (µM/plant/h) of rice cv. Bas-386 under field conditions

23(a) 15 DAT

Strains (C)	Nitrogenase activity				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	2.02	2.31	1.15	2.60	2.02
SRR-1	5.78	8.69	6.51	7.67	7.17
S1	4.63	5.21	5.79	7.24	5.72
ZB-SK-5	4.05	5.65	4.92	6.65	5.32
Mean(N)	4.79		5.34		
Mean(2,4-D)	4.36(0.0ppm)		5.76(3.0ppm)		

CD(5%)** **B**-0.65 **C**-0.91

23(b) 30 DAT

Control	7.24	7.82	7.52	9.55	8.04
SRR-1	17.37	19.98	16.17	21.72	18.82
S1	14.77	15.89	17.37	17.37	15.92
ZB-SK-5	14.47	15.64	16.79	14.18	15.27
Mean(N)	14.15		15.09		
Mean(2,4-D)	13.97		15.27		

CD(5%)** **A**-0.83 **B**-0.83 **C**-1.18 **BC**-1.67 **ABC**-2.35

23(c)At the time of harvesting

Control	14.19	16.64	13.60	14.99	14.84
SRR-1	22.58	27.50	20.85	23.16	23.53
S1	19.11	20.27	20.55	22.58	20.64
ZB-SK-5	16.22	16.22	14.47	14.77	15.42
Mean(N)	19.09		18.10		
Mean(2,4-D)	17.69		19.77		

CD(5%)** **B**-1.02 **C**-1.44 **AC**-2.04

** CD (5%) for all other treatments/interactions is NS

Table 24 (a, b, c). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on root nitrogenase activity (µM/plant/h) of rice cv. PR-116 under field conditions

24(a) 15 DAT

Strains (C)	Nitrogenase activity				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	2.02	2.46	2.46	2.61	2.39
SRR-1	6.08	8.97	6.65	9.12	7.71
S1	4.05	4.34	4.92	5.78	4.78
ZB-SK-5	6.95	7.24	5.93	7.94	6.84
Mean(N)	5.26		5.59		
Mean(2,4-D)	4.89(0.0ppm)		5.97(3.0ppm)		

CD(5%)** **B**-0.73 **C**-1.03

24(b) 30 DAT

Control	3.47	10.43	8.68	10.27	9.30
SRR-1	26.64	26.93	27.22	25.77	26.64
S1	15.92	17.08	16.21	19.98	17.30
ZB-SK-5	19.68	24.32	21.14	26.93	23.02
Mean(N)	18.61		19.53		
Mean(2,4-D)	17.92		20.22		

CD(5%)** **B**-1.37 **C**-1.95 **BC**-2.75

24(c)At the time of harvesting

Control	18.24	21.42	19.39	24.03	20.78
SRR-1	29.24	30.69	28.67	32.14	30.19
S1	29.24	23.74	23.46	27.22	21.28
ZB-SK-5	25.77	28.09	25.19	29.54	27.15
Mean(N)	24.79		26.20		
Mean(2,4-D)	23.89		27.14		

CD(5%)** **A**-1.22 **B**-1.22 **C**-1.73

** CD (5%) for all other treatments/interactions is NS

Table 25. **Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on paranodule number of rice cv. Bas-386 under field conditions at time of harvesting.**

Strains (C)	Paranodule number				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	0.00	2.00	1.33	3.00	1.58
SRR-1	13.33	12.66	8.66	9.00	10.92
S1	12.00	13.00	7.00	9.33	10.33
ZB-SK-5	10.00	9.00	7.33	8.00	8.58
Mean(N)	9.00		6.71		
Mean(2,4-D)	7.46(0.0ppm)		8.25(3.0ppm)		

CD(5%)** **A**-1.12 **C**-1.58 **AC**-2.23

** CD (5%) for all other treatments/interactions is NS

Table 26 (a, b). Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on paranodule number of rice cv. PR-116 under field conditions

26(a) 15 DAT

Strains (C)	Paranodule number				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	0.00	1.00	0.66	2.00	0.92
SRR-1	6.00	8.00	5.66	6.00	6.42
S1	3.00	4.66	3.33	4.66	3.92
ZB-SK-5	5.00	5.00	5.33	4.66	5.00
Mean(N)	4.08		4.04		
Mean(2,4-D)	3.63(0.0ppm)		4.50(3.00ppm)		

CD(5%)** **B**-0.86 **C**-1.23

26(b) 30 DAT

Control	1.66	2.66	1.00	1.66	1.75
SRR-1	4.66	6.33	3.66	6.00	5.16
S1	2.33	5.00	3.00	4.33	3.66
ZB-SK-5	4.33	6.00	4.33	3.66	4.58
Mean(N)	4.13		3.46		
Mean(2,4-D)	3.13		4.46		

CD(5%)** **A**-0.66 **B**-0.66 **C**-0.94

** CD (5%) for all other treatments/interactions is NS

Plate VI. **Effect of inoculation of *A. caulinodans* (SRR-1) and 2,4-D on paranodule formation of rice roots cv. Bas-386 under field conditions**

a. Uninoculated
b. Inoculated + 2,4-D

Plate VII. **Effect of inoculation of *A. caulinodans* (SRR-1) and 2,4-D on paranodule formation of rice roots cv. PR-116 under field conditions**

Plate VI

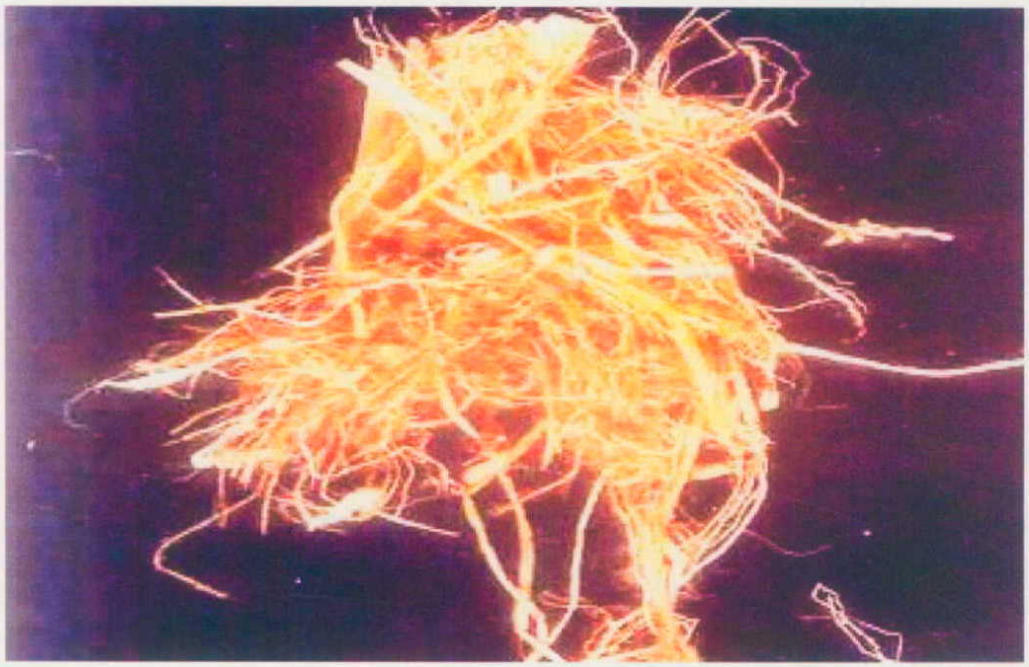

Plate VII

Paranodules were observed only upto 30DAS but not during the reproductive stages. This may be due to the labile/vulnerable stage of the transplanted rice plants. After that period the root surface becomes more tough and thus reduced the chances of crack entry. Nie (1983) reported that 2,4-D can operate only on young, lateral or branch rice roots and thus paranodules will revert back to original roots at the harvesting stage.

2,4-D addition may account for more crack entry events, more number of lateral rootlets and more number of bacteria invading the epidermal and outer cortical cells intercellularly. Thus this contributes to increased paranodule number as well as increased nitrogenase activity. Van-Nieuwenhave *et al* (2000) also reported low nitrogenase activity of the rice roots inoculated with *A. caulinodans* under wetland conditions.

4.3.9 Tiller number

Bas-386 : The average tiller number varied from 8-13. The ¾ N level plots produced significantly more number of tillers over the full N level. The tiller number enhanced significantly among plots by the 2,4-D treatment. Similarly, all azorhizobial strains performed significantly better over the control. The maximum tiller number was observed in SRR-1 followed by S_1 (Table 27).

PR-116 : The average tiller number varied from 10-18. N application was not found to be correlated with number of tillers per plant. On the other hand, the 2,4-D applied plots produced significantly

Table 27. **Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on tiller number of rice cv. Bas-386 under field conditions at the time of harvesting**

Strains (C)	Tiller number pl^{-1}				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	7.53	8.93	7.93	10.03	8.61
SRR-1	13.76	14.66	11.03	13.23	13.17
S1	12.10	13.00	10.13	10.80	11.51
ZB-SK-5	10.93	11.23	8.70	10.83	10.42
Mean(N)	11.52		10.34		
Mean(2,4-D)	10.26(0.0ppm)		11.59(3.0ppm)		

CD(5%)** **A**-0.32 B-0.32 **AB**-0.46 **C**-0.46 **AC**-0.65

** CD (5%) for all other treatments/interactions is NS

more number of tillers. The cultures responded significantly better over the control. SRR-1 gave best results followed by S_1 (Table 28).

4.3.10 Grain weight

Bas-386 : The average grain weight per plant ranged from 7.1-13.4 g. The grain weight increased with increasing dose of N level. Similarly, the grain weight enhanced significantly by the 2,4-D treatment and by application of *Azorhizobium* strains over the control. The maximum grain weight was recorded by SRR-1 followed by S_1 (Table 29).

PR-116 : The average grain weight per plant ranged from 18.1-31.7 g. The mean value of both the N-levels were found to be statistically at par amongst themselves. Whereas, the grain weight showed significant increase by application of 2,4-D and *Azorhizobium* strains. All cultures performed better over the control. The best performer was SRR-1 followed by reference strain S_1 (Table 30).

4.3.11 Yield

Bas-386 : The average range of yield was observed from 4.4-6.5 q/acre. No significant difference was observed between both the N-levels. Whereas the application of 2,4-D and *Azorhizobium* strains increased the grain yield significantly over the control (Plate VIII). The maximum yield was observed by SRR-1 followed by reference strain ZB-SK-5 (Table 31).

Table 28. **Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on tiller number of rice cv. PR-116 under field conditions at the time of harvesting**

Strains (C)	Tiller number pl^{-1}				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	10.76	11.43	9.83	9.83	10.46
SRR-1	16.50	17.96	15.76	21.36	17.90
S1	14.38	14.76	12.76	17.53	14.86
ZB-SK-5	11.46	12.83	10.60	15.36	12.56
Mean(N)	13.76		14.13		
Mean(2,4-D)	12.76(0.0ppm)		15.14(3.0ppm)		

CD(5%)** **B**-0.59 **AB**-0.83 **C**-0.83 **AC**-1.18
BC-1.18 **ABC**-1.66

** CD (5%) for all other treatments/interactions is NS

Table 29. **Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on plant grain weight (g) of rice cv. Bas-386 under field conditions at the time of harvesting**

Strains (C)	Grain weight pl^{-1}				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	5.62	6.71	7.52	8.42	7.06
SRR-1	10.92	14.15	13.43	15.05	13.39
S1	10.13	12.17	12.46	14.44	12.30
ZB-SK-5	9.13	11.96	10.92	12.94	11.24
Mean(N)	10.09		11.89		
Mean(2,4-D)	10.02(0.0ppm)		11.98(3.0ppm)		

CD(5%)** **A**-0.38 **B**-0.38 **C**-0.54 **BC**-0.76

** CD (5%) for all other treatments/interactions is NS

Table 30. **Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on plant grain weight (g) of rice cv. PR-116 under field conditions at the time of harvesting**

Strains (C)	Grain weight pl^{-1}				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	17.51	19.30	16.92	18.64	18.09
SRR-1	29.49	32.82	31.52	33.19	31.75
S1	27.25	31.81	27.10	32.05	29.55
ZB-SK-5	22.13	26.93	23.95	28.49	25.38
Mean(N)	25.90		26.48		
Mean(2,4-D)	24.48(0.0ppm)		27.91(3.0ppm)		

CD(5%)** **A**-0.40 **B**-0.40 **C**-0.57 **AC**-0.80 **BC**-0.80

** CD (5%) for all other treatments/interactions is NS

Table 31. **Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on yield (q acre^{-1}) of rice cv. Bas-386 under field conditions**

Strains (C)	Yield				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	3.93	4.43	4.76	4.50	4.41
SRR-1	5.71	6.73	6.14	7.61	6.54
S1	4.47	5.14	4.76	5.94	5.08
ZB-SK-5	5.19	6.80	4.81	6.73	5.88
Mean(N)	5.30		5.66		
Mean(2,4-D)	4.97(0.0ppm)		5.98(3.0ppm)		

CD(5%)** **B**-0.042 **C**-0.59

** CD (5%) for all other treatments/interactions is NS

Plate VIII **Effect of inoculation of *A. caulinodans* and 2,4-D on rice cv. Bas-386 under field conditions**

Plate VIII

PR-116 : The average range of yield was observed from 27.2-33.4 q/hac. The difference in yield of both the N-levels was found to be non significant thus indicating that yield was irrespective of the N-added. The significant increase was observed in yield of plots by application of 2,4-D and by use of all strains of *Azorhizobium* over the control (Plate IX). While the strains were found to be statistically at par amongst themselves. The maximum yield was produced by SRR-1 followed by reference strain S_1 (Table 32).

The rhizobial inoculation may change the physiological status of rice which enable the plant to make more efficient use of fertilizer-N inputs for seed production and thus favour improved grain filling. The increase in total biomass by rice seedlings following rhizobial inoculation may account for increase in tiller number which was also reflected in higher grain yield.

Yanni *et al* (1997) reported that rhizobial inoculation increased the grain yield of rice by 10-45% over a wide range of N supply in a field experiment. Biswas *et al* (2000a, b) reported increased grain yield at different N rates following rhizobial inoculation. Peng *et al* (2002) reported 16% increase in grain yield by rhizobial inoculation which may be due to increase in total biomass production rather than harvest index.

Table 32. **Effect of inoculation of *Azorhizobium caulinodans* strains, 2,4-D and N fertilizer on yield (q ha^{-1}) of rice cv. PR-116 under field conditions**

Strains (C)	Yield				Mean
	N-levels (A)				
	¾ N		N(Full)		
	2,4-D (ppm) (B)				
	0.0	3.0	0.0	3.0	
Control	25.34	29.18	27.12	27.80	27.22
SRR-1	32.19	36.07	30.55	34.66	33.38
S1	31.78	33.69	31.92	35.61	33.28
ZB-SK-5	30.55	31.51	29.18	31.92	30.82
Mean(N)	31.31		31.05		
Mean(2,4-D)	29.76(0.0ppm)		32.59(3.0ppm)		

CD(5%)** **B**-2.15 **C**-3.06

** CD (5%) for all other treatments/interactions is NS

Plate IX. Effect of inoculation of *A. caulinodans* and 2,4-D on rice cv. PR-116 under field conditions

a. Uninoculated
b. Inoculated + 2,4-D

Plate IX

4.3.12 Competitiveness of inoculated strains

The paranodules or the young lateral roots of the plants treated with different *Azorhizobium* strains were studied for their establishment using antibiotic resistance marker. All the nodules/root segments were found to be colonized with the culture with which these were treated showing thereby 100% establishment of the respective cultures of respective treatments. On the other hand the control plants were found to be having no establishment of the native *Azorhizobium caulinodans* strains indicating thereby that the soil in which the experiment was conducted is devoid of these bacteria and it also authenticates that the establishment of *Azorhizobium* strains is the result of the treatment.

Chapter V

SUMMARY

The extension of symbiosis like systems to non-leguminous crops as rice would curtail the use of chemical fertilizers that have profound detrimental effects on the agro-ecological systems. The inoculation of cereals with various non rhizobial diazotrophic bacteria has been undertaken with the expectation that they would establish themselves intercellularly within the root system, fix nitrogen endophytically and provide combined N for enhanced crop production. Among diazotrophs, *Azorhizobium caulinodans* that forms roots and stem nodules on *Sesbania rostrata*, a typical legume, has the potential of contributing towards rice production due to its ability to invade lateral roots by crack entry and this formed the basis of present investigation.

A total of ten *Azorhizobium caulinodans* isolates were isolated from root as well as stem nodules of *Sesbania rostrata*. The maximum *in vitro* nitrogenase activity was recorded by root isolate SRR-1 followed by stem isolate SRS-3. Based on the nitrogenase activity, SRR-1 was selected as better performer and thus used for further studies. SRR-1 and two reference strains S_1 and ZB-SK-5 were used to study along with phytohormones for induction of paranodules and nitrogen fixation in the

roots of rice cv. Bas-386 and cv. PR-116. The root isolate SRR-1 and SRR-5 exhibited resistance to maximum number of antibiotics used.

Under laboratory conditions, the inoculation of *Azorhizobium caulinodans* alone showed increase in root length, shoot length, fresh weight, dry weight, paranodule and lateral rootlet number and nitrogenase activity over the uninoculated control. The phytohormone treatment alone decreased all the parameters except the lateral rootlet number. However, the dual treatment of *A. caulinodans* strains and phytohormones nullified the adverse effect of kinetin and GA_3 treatment alone. The best performer among all *Azorhizobium* strains was root isolate SRR-1. Among the two phytohormones kinetin proved better over GA_3. The rice seedlings of cv. PR-116 was found to be comparatively better than cv. Bas-386 for all parameters except root and shoot length.

In the field trial of rice cv. Bas-386 and cv. PR-116 a significant increase was observed in all the parameters studied with all strains of *Azorhizobium* over the uninoculated control at almost all stages of sampling. The 2,4-D treatment along with *Azorhizobium* strains showed encouraging results for paranodulation, nitrogenase activity, yield and yield parameters. Saving in the input of N fertilizer besides improving yield. The establishment of *A. caulinodans* strains was found to be 100% in both the varieties of rice.

The induction of nodulation via crack entry by *A. caulinodans* has provided excellent scope in terms of N transfer to rice crop for higher

productivity. This raises the possibility of using *A. caulinodans* as a future biofertilizer for the non-legume crops which would be a boon to fulfill the requirements of N through BNF.

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