

INTERACTION OF AZORHIZOBIA WITH
Oryza sativa

Dissertation

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

DOCTOR OF PHILOSOPHY
In
MICROBIOLOGY
(Minor Subject : Biochemistry)

By

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CERTIFICATE I

This is to certify that the thesis entitled “**Interaction of azorhizobia with *Oryza sativa***” submitted for the degree of **Ph.D.**, in the subject of Microbiology (Minor subject: Biochemistry) of the **Punjab Agricultural University, Ludhiana**, is a bonafide research work carried out by **Anu Kalia** (L-98-BS-81-D) 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.



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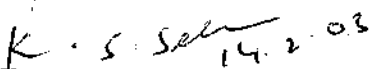
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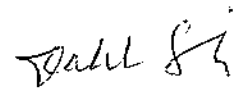
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This is to certify that the thesis entitled "**Interaction of azorhizobia with *Oryza sativa***" submitted by **Anu Kalia** (L-98-BS-81-D) to the **Punjab Agricultural University, Ludhiana**, in partial fulfillment of the requirements for the degree of Ph.D. in the subject of Microbiology (Minor subject : Biochemistry) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.


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"Accept what comes to you totally and completely so that you can appreciate it, learn from it, and then let it go because intentions automatically seek their fulfillment if left alone"

(Deepak Chopra)

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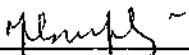
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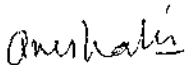
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ABSTRACT

Six isolates of *Azorhizobium caulinodans* isolated from root and stem nodules of *Sesbania rostrata* possessed specific intrinsic antibiotic resistance spectra and nitrogenase activity with stem isolates showing better nitrogen fixing potential under free-living conditions. The isolates were screened for further studies on *S. rostrata* and their inoculation resulted in increase in plant height, fresh biomass, nodule number and mass and root nitrogenase activity both under pot and field conditions with S₂ isolate giving the best performance. After screening four isolates were used for the lab studies on paranodule induction, intercellular incorporation and nitrogen fixation using chemicals 2,4-D, NAA, Naringenin and Acetosyringone. Isolate S₁ gave best results at 1.5ppm, 3.0 ppm and 10⁻⁵ M concentration of the former three respectively and R₄ at 400ppm concentration of Acetosyringone regarding root-shoot length, paranodule induction and nitrogen fixation. Study of the stained microtomic sections revealed accumulation and intercellular incorporation of azorhizobia in the cortical cells with 2,4-D treated roots showing the best results. Field experiment for rice cv PR-115 using the four isolates gave results of increased plant height, biomass, grain weight and yield in *Sesbania* buried plots as it supplements soil by providing micronutrients and biomass and also due to nodule induction and nitrogen fixation by the rice root itself that resulted in direct transfer of nitrogen to the plant resulting in increased N-economy of the rice plants.


Signature of Major Advisor


Signature of Student

Key Words: *Azorhizobium*, paranodulation, non-legumes, signal compounds, auxins

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Chapter 1

INTRODUCTION

Nitrogen is the vital and key element governing plant productivity because 1.8 to 2.0 kg Nitrogen is required to produce 100 kg of grain for rice (Patnaik and Rao, 1979). Cereal crops as rice and wheat plunder minerals as nitrogen, phosphorus and several other micronutrients from the soil. 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.

Despite the heavy use of nitrogen fertilizers, soil nitrogen and biological nitrogen fixation (BNF) remain primary sources for a vast area under rice production. Moreover, the concept of sustainability has profoundly catalyzed a re-evaluation of agricultural development in the past few decades. Agricultural systems are assessed not only in terms of inputs and outputs but also in context of their environmental and social impacts. Many approaches and philosophies contribute to vision of agricultural sustainability, but one theme remains consistent i.e. the crucial nitrogen management.

Few agrotechnologies meet the criteria for sustainable agricultural development as neatly as the legume BNF (Gupta and Pandher, 1996; Kannaiyan, 1995). These considerations have stimulated the research on alternatives, such as biological nitrogen fixation. BNF has been estimated to contribute more than 170 million tons of fixed nitrogen to the biosphere. 80% of

stable biologically fixed nitrogen is a direct result of the symbiotic interaction of members of Rhizobiaceae and some actinomycetes with leguminous as well as certain non-leguminous plants. (deBruijn *et al*, 1995).

Better mineral utilization could be obtained by integrated use of organic and fertilizer nitrogen. Organic nitrogen could be obtained through several agronomical practices. The symbiotic association of endophytic BGA *Anabaena azollae* with water fern *Azolla* can fix 30 –60 kg N ha⁻¹ per rice yield (Kumarasinghe and Zapata, 1986). Incorporation of *Azolla*-*Anabaena* to paddy soils increases rice yield (Singh, 1977). Badaway *et al* (1998) observed that growth and grain yield of rice cv. Riho was improved considerably by using *Azolla*. Similarly, increase in yield by 14 % of rice over control was recorded by Roger and Kulasooriya (1980). *Anabaena azollae* immobilized in polyurethane foam produced ammonia under field conditions (Kannaiyan *et al*, 1997) Thus blue-green algae have established their potential as a biofertilizer. (Suri and Puri 1994, Gupta *et al*, 1997).

Legume BNF though may not be a panacea, but it is often the most feasible and economical nitrogen input for resource poor farmers. Legumes can be used as green manure as legume-rhizobia symbiosis can contribute up to 400 kg N ha⁻¹. Among several legumes used as green manure *Sesbania rostrata* has recently gained popularity, as it is a fast growing tropical legume with high biomass capacity and higher nitrogen fixation rates and a unique characteristic of formation of aerial stem nodules. (Kannaiyan and Kaldurai, 1995). The root and stem nodules of *S. rostrata* are inhabited by *Azorhizobium caulinodans* (Dreyfus

et al, 1988). Kannaiyan (1995) observed a significant increase in rice yield upon incorporation of *Sesbania rostrata* biomass at 20 tons ha⁻¹.

Associative nitrogen fixation is the arena where considerable amount of research efforts are being poured in as nitrogen fixed by BNF if made directly available to plant could provide benefits to some extent of rhizobia-legume symbiosis. Wetland or lowland rice fields showed that certain bacteria get associated with rice roots and basal part of shoot i.e. rhizocoenosis, can fix nitrogen (Kannaiyan, 1998; Watanabe, 1986). Experimental rhizobial inoculation influences seedling vigour and yield of rice. (Biswas *et al*, 2000).

Formation of nodule-like structures (paranodules) in cereal crops could be obtained by several treatments such as use of hydrolytic enzymes along with polyethylene glycol/ Calcium Chloride treatment (Al-Mallah *et al*, 1989), direct inoculation by several rhizobia (Ladha and Huang, 1997), use of growth regulators as synthetic auxins 2,4-D, NAA, cytokinin (Nie, 1993) or use of signal phenolic compounds such as the flavonoids, isoflavones and flavanols. (Jain and Jain, 1998).

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 enzyme treatment, invaded by diazotrophs that increase the nitrogen uptake by plant.

Stem colonizing *A.caulinodans* invades rice by 'crack entry' and colonize nodular structures (Kennedy *et al*, 1997) while *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). Four families of flavonoids have the ability to induce nod-genes (Denarie *et al*, 1992). Flavonoids are produced both by legumes and non-legumes. Naringenin acts as most powerful inducer of nod genes of *A. caulinodans* (Goethals *et al*, 1989) and may act as a signal molecule for nod gene independent intercellular colonization of rice and wheat by *A. caulinodans* ORS571 (Webster *et al*, 1997). Naringenin at 10^{-4} to 10^{-5} M concentrations stimulated colonization of lateral root cracks (LRC) of wheat and rice by ORS571 (pXLGDA) (Webster *et al*, 1997). Naringenin and *A. caulinodans* caused significant increase in seminal and adventitious roots of rice (Gopalaswamy, 1998).

Kannaiyan *et al* (1998) showed xylem colonization of rice roots following entry at lateral root cracks by *A.caulinodans*. ORS571. 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. *A. caulinodans* xylem colonization in rice roots is also confirmed by Gopalaswamy *et al* (2000). Stone *et al* (2001) also reported the xylem colonization of *A.caulinodans* ORS571 of the *Arabidopsis thaliana* roots.

Following are objectives of present study

- a) Isolation of *A. caulinodans* from stem and root nodules of *Sesbania rostrata*.
- b) Purification and characterization of isolates and to study the antibiotic resistance spectra and nitrogen fixation ability of *Azorhizobium* isolates using antibiotic solutions of known concentrations.
- c) To study the effect of NAA, 2,4-D and flavonoid on formation of rootlets and paranodules in rice roots under lab conditions.
- d) To study effect of inoculation of selected strains of stem and root isolates of *Azorhizobium* on *Sesbania rostrata*.
- e) To study effect of incorporation of both the inoculated and uninoculated *S. rostrata* along with control on rice growth, nitrogen fixation and establishment of *Azorhizobium*.

Chapter-II

REVIEW OF LITERATURE

Rice, the staple food crop for about 3 billion people, is actually cultivated in areas where the population pressure is the highest in the world accompanied by scarce land and agricultural resources which are the prime factors that hunchback rice productivity (Kannaiyan and Kalidurai, 1995). The importance of commercial nitrogenous fertilizers for maintaining crop productivity in agriculturally advanced or less advanced countries is unquestioned (Chitra and Kannaiyan, 1995). Thus replenishment of soil nitrogen in modern agriculture most commonly involves extensive application of chemical fertilizers (Peoples *et al*, 1995)

Rice recovers about 30-40% of applied nitrogen. Patnaik and Rao (1979) reported that rice plant needs to absorb about 19-21 Kg N to produce one ton of brown rice. Accordingly, the rice plant must absorb a large amount of nitrogen to produce higher yield. But urea the most common nitrogen source in developing countries is the most energy intensive fertilizer (Mudahar and Hignett, 1982). Moreover, excessive or inefficient application of chemical fertilizers suffers from serious drawbacks such as severe negative environmental impacts as it is difficult to match nitrogen supply to actual requirements of a crop at a given ecosite and any excess reduced nitrogen (ammonium) in agricultural or forest ecosystems may lead to acidification through process of nitrification, if significant leaching of nitrate occurs (Kennedy, 1986; 1991).

Rice has been cultivated over 143 million hectares of land in world and 75% of this is planted as wetland rice. Wetland ecosystems suffer from significant nitrogen loss by ammonia volatilization, leaching, denitrification and run off (George *et al*, 1992) than other crop ecosystems (Kannaiyan, 1992).

The search for alternatives or supplements to fossil fuel-based inorganic nitrogen fertilizers have been brought about not only by the likelihood of future price increase for chemical fertilizers but also by the need to maintain long term soil productivity and ecological sustainability. The concept of sustainability has profoundly catalyzed re-evaluation of agricultural systems which are now assessed not only in terms of inputs and outputs but also in context of their environmental and social impacts. The global vision of sustainable land use involves crucial management of nitrogen and other nutrients that determine soil health and texture.

All philosophies, practices and approaches stand nowhere except the use of systems that are capable of fixing their own nitrogen i.e. biological nitrogen fixation. Intrinsically, BNF symbolizes and explains sustainability as it exploits less its own environment, is inexpensive, renewable does not pollute or may even provide a positive contribution to its environment. BNF has been estimated to contribute more than 170 million tons of fixed nitrogen to the biosphere (Earl and Ausubel, 1993).

The additional nitrogen requirement could be met by increasing the levels of BNF. The utilization of nitrogen fixing leguminous green manure crops for rice production could be a viable component of integrated nutrient management to

enrich soil organic matter (JiaoBin, 1983) and to attain higher grain yield (Ladha *et al*, 1988). Green manures can sustain lowland rice productivity and reduces farmer's dependence from mineral nitrogen fertilizers and high cash yield up to 50 kg N ha⁻¹ crop⁻¹ (Roger and Ladha, 1992). In lowland rice field ecosystem, free-living and plant associated diazotrophs contribute nitrogen by continuous BNF to produce 2-3 t grain ha⁻¹ (Ladha *et al.*, 1993) Wetland conditions favour higher BNF than dryland rice conditions. Thus the benefits of green manure addition remain limited. Researches are cajoled by the very idea of additional nitrogen inputs through BNF to non-legumes, 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 BIOFERTILIZERS FOR RICE

Biofertilizers for rice basically involved the addition of organic nitrogen obtained through use of green manures, induction of legume-rhizobial symbiosis-like process (induced nodulation) or use of associative nitrogen fixers in dual or multiple inoculations.

2.1.1 GREEN MANURES:

Few agrotechnologies meet the criteria for sustainable agricultural development as neatly as legumes BNF. Green manuring particularly with nitrogen fixing leguminous plants could add low cost substantial amount of organic nitrogen to rice besides improving soil health, texture and properties (Jiao, 1983), recycle nutrients (Abrol and Palaniappan, 1988), conserve nitrate NO_3 mineralized during dry fallow between two rice crops (Pandey and Morris, 1983) and increases microbial population in rice soils (Ferriera and Raghu, 1981). Green manure amendment has been reported to reduce the incidence of bacterial disease in rice (Premlatha Dath, 1981), sheath blight disease of rice (Kannaiyan and Prasad, 1981; Kannaiyan, 1987) and residue levels of persistent pesticides in rice (Ferreira and Raghu, 1981). Of the different kinds of nitrogen fixing leguminous green manures plants *S. aculeata* and *Crotolaria juncea* were widely grown in Asian rice farming systems for green manure value and were well known to contribute an average of $2.6 \text{ kg N ha}^{-1} \text{ day}^{-1}$ (Ladha *et al*, 1988). Rising interest in nitrogen fixing legumes led to hitherto unknown leguminous plants that have green manure value for rice crop (Rinaudo *et al*, 1983; Meelu and Morris, 1988). Hagerup (1928) first reported the phenomenon of stem nodulation in *Aeschynomene spp*. More interest in this process aggravated after discovery of another Stem nodulating plants viz. *Sesbania*, *Aeschynomene spp*. (Dreyfus and Dommergues, 1981) and *S. rostrata* (Rinaudo *et al*, 1983; Kannaiyan, 1992) growing as wild plants under water logged soils in Senegal (Africa) (Dreyfus *et al*, 1988) were recommended as potential green manure

plants that produce stem nodulation (Kalidurai and Kannaiyan, 1989). *S. rostrata* is known to tolerate waterlogging and high soil nitrogen conditions (Becker *et al*, 1986) and produce biomass around 25 t ha⁻¹ in 45 days under rice field conditions (N'doye *et al*, 1994). Ladha *et al* (1987) reported production 20-30 t ha⁻¹ biomass of *S. rostrata* under water logged conditions in fallow rice field with in 40-60 days duration. Rinaudo *et al* (1988) reported that BNF contributes 50-70 % to the total nitrogen accumulation by *S. rostrata* in West Africa. In India it has been reported to contribute 40-60 N ha⁻¹ for rice crop per season and significantly increased grain yield of rice (Kannaiyan *et al*, 1987). Application of phosphorus, nitrogen and organic matter stimulates nodule induction, nitrogen fixation and biomass production in leguminous green manure crops (Gibson *et al*, 1982; Balasubramani and Kannaiyan, 1991). When incorporated in soil, under favorable conditions, decomposition of *S. rostrata* occurs and thus nitrogen mineralization becomes rapid (Shi Shu-Lian *et al*, 1981) leading to accumulation of ammoniacal, nitrite and nitrate (Kalidurai and Kannaiyan, 1988).

Stem nodulation is a fascinating biological system and has attracted the attention of research workers both in basic sciences and applied sciences. Stem nodules in *S. rostrata* are produced on pre-determined nodulation sites on stem (Dreyfus *et al*., 1984; Kannaiyan, 1995) which are always the 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* (Schade, 1940; Arora, 1954; Becker *et al*, 1990; Balasubramani and Kannaiyan, 1992). Native population of *Azorhizobium* in soil

remains at low level and thus seeds and aerial parts (leaves, flower parts) seem to be the mode of initial entry of bacteria in soil (Kannaiyan, 1989; Ladha *et al.*, 1988).

Stem nodules are produced on aerial portion of stem above the ground where occurs an obvious advantage of negligible competition with other bacteria. These stem nodules have the surrounding cortical cells, which are capable of carrying out photosynthesis as they harbor chloroplasts and are so called 'self-energy generating system'. (Fleischmann, 1991). Ladha *et al* (1990) has reported that *Azorhizobium* contains bacteriochlorophyll and photosynthetic reaction center.

Stem nodules possess 3-4 times more nitrogen fixing potential than root nodules (Balasubramani *et al* 1992). Dreyfus *et al* (1984) reported *S. rostrata* stem nodule nitrogenase activity of about 600 $\mu\text{mole C}_2\text{H}_4 \text{ h}^{-1} \text{ plant}^{-1}$ lowland rice condition. Defoliation reduces root activity whereas it had no effect on activity of stem nodules which possess nitrogenase activity for a longer time in illuminated conditions and more over flooding causes root nodule decay and decrease in nitrogen fixing ability of root nodule decay and decrease in nitrogen fixing ability of root nodules (Manguiat *et al*, 1987). Seed and foliar inoculation induces stem nodulation (Chitra and Kannaiyan, 1994) while increases humidity induces only early nodule appearance in *S.rostrata* (Patel *et al*, 1996).

The stem nodulating rhizobia of *S. rostrata* were reclassified by DNA-RNA hybridization and protein gel electrophoresis to a single genus *Azorhizobium* having a single species *A. caulinodans* (Dreyfus *et al*, 1988). The bacteria moves

inside by crack entry and reaches pericycle of nodule primordia penetrates intracellular spaces and multiplies concurrently with meristematic activity of cells (Dreyfus *et al*, 1983) and forms branched infection threads (Tsien *et al*, 1983).

Balasubramani and Kannaiyan (1991) reported insect transmission of bacteria as it was found to be present on Blister beetle. Epiphytic survival and insect transmission seem to be basic techniques of inoculation of soil with bacteria (Ladha *et al*, 1989). The rainwater washes away the epiphytically growing rhizobia to infection sites on stem causing nodulation (Becker *et al*, 1990). However, stem nodules release Azorhizobia upon senescence, which may survive saprophytically in soil. Ladha *et.al* (1989) have reported that Azorhizobia released from stem nodules were able to survive in flooded soils and in rhizosphere and histosphere of wetland rice.

2.1.2 FREE-LIVING DIAZOTROPHS IN RICE RHIZOCOENOSIS

Several free-living diazotrophs including a diverse range of microbial species are capable of nitrogen fixation (Mulder and Brotonogoro, 1974; Postgate, 1972). Wet flooded conditions favour BNF by providing.

- a) Photo-oxic floodwater and surface soil for phototrophic, free-living or symbiotic blue-green algae (Roger and Ladha, 1992; Kannaiyan, 1995).

The potential growth of this nitrogen fixing system may be exploited to harness atmospheric nitrogen to increase crop productivity (Kannaiyan, 2001).

- b) Aphotic –anoxic soil for anaerobic or microaerobic, heterotrophic bacteria.

Thus rice plant provides two major sub-environments i.e-submerged plant parts and roots. Roots support the growth of bacteria outside and inside the roots. The rhizosphere is oxidised by oxidising ability or oxygen excretion. When the rhizosphere cannot maintain itself in an oxidized state then it become reduced and substances as sulphides accumulates. This favours various microorganisms such as *Azotobacter*, *Clostridium*, *Drexia* and *Beijerinckia* to pioneer the colonization of 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 (Venkatraman, 1979; Kennedy and Tchan, 1992). Singh *et al* (1999,2000) reported increase in yield and yield attributes and nitrogen uptake in wheat by *Azotobacter* inoculation. An analogue-resistant mutant of *Azotobacter chroococcum* strain A 103 when inoculated in wheat caused increase in grain yield (Lakshminarayana *et al*, 2000) Kannaiyan *et al* (1980) reported positive influence of foliar spray of *A. chroococcum* in rice and found significant increase in grain and straw yield. Several processes other than nitrogen fixation could account for positive effects, including production of growth regulators protection from root pathogens and modification of nutrients uptake by the plant (Tchan, 1988).

2.1.2 ASSOCIATIVE DIAZOTROPHS IN RICE RHIZOSPHERE

A large proportion of total microbial activity in soil is likely to be associated with growth of plants or their subsequent breakdown as in greatest abundance. For associative nitrogen-fixers, Dobereiner (1989) has stressed that too much may be assumed about the degree of an associative relationship simply because

bacteria are located in rhizosphere near the root surface. Certain bacterial species show associations with several non-leguminous plants such as *Azospirillum brasilense* which penetrates the roots of graminaceous plant species, grow in rhizosphere and intercellularly (Reinhold and Hurek, 1989; Christiansen-Weniger and VanVeen, 1991; Foster and Rovira, 1976), *Acetobacter diazotrophicus* in root systems of sugarcane (Li and MacRae, 1991), *Pseudomonas diazotrophicus* in root systems of sugarcane (Li and MacRae, 1991) forming 80% of total bacterial population associated with rice roots (Barraquio *et al* 1983; Watanabe *et al*, 1987), *Alcaligenes faecalis* in rice roots (You and Zhou, 1989), *Klebsiella pneumoniae* in root system of sugarcane (Li and MacRae, 1991), *Klebsiella oxytoca* and *Enterobacter cloacae* with rice (Fuji *et al*, 1987; Yoo *et al*, 1986).

Creus *et al* (1997, 1998) observed better tolerance of *Azospirillum* inoculated wheat seedlings under osmotic and salt stress conditions. Didonet and Magalhaes (1993) observed significant promotion of plant growth in wheat by *Azospirillum* inoculation and thus being recommended as biofertilizer for wheat. (Sandhu, 2001). *Azospirillum* inoculation increases nitrogen content of rice (Nayak *et al*, 1986). Panwar and Singh (2000) observed increase in leaf surface area, Chlorophyll concentration, total biomass and grain yield in wheat upon seed inoculation by *Azospirillum* and *Bacillus*.

2.1.3 OTHER DIAZOTROPHS OF SIGNIFICANCE

Genus *Rhizobium* includes diverse kind of diazotrophs existing in free living state in soil and in rhizosphere of legumes as well as non- legumes and upon

entrance in host roots show development of nodules or nodule-like structures in legumes (Allen *et al*, 1953; Wilde, 1951) and non-legumes such as *Parasponium*. Chaintreuil *et al* (2000) reported that bacteria of genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Allorhizobium* and *Mesorhizobium* elicit nodule formation in legumes and non-legumes. *Rhizobium parasponium* RP-501 and *Bradyrhizobium* CCP-293 induces nodulation in oil seed rape (Cocking *et al*, 1992). *B. Parasponium* infects oil seed rape (*Brassica napus*) without enzyme treatments (Cocking *et al*, 1990) and produced variety of nodule types of which some contained red/pink pigmentation suggesting but not proving the production of leghaemoglobin.

Vij and Chopra (2000) reported that rhizobia of *Parasponium*, stem nodules of *Aeschynomene* and *Sesbania sp.* infect root system of rice, wheat and maize by entry through cracks. Ladha and Huang (1997) reported clear morphological changes in root roots upon rhizobial inoculation. The root hairs were deformed and curling was observed at 90 to 180° angles after 2-3 days of *Rhizobium* inoculation. Biswas *et al* (2000) have observed increase in uptake of Nitrogen, Phosphorus and Potassium by 10-28% upon rhizobial inoculation in low land rice fields. Inoculation also increased iron uptake by 15-64% and accumulation of IAA in external root environment of rice. Biswas *et al* (2000) observed that *Rhizobium* inoculation influences seedling vigor and yield of rice. *R. leguminosarum* bv *trifolii* E-11 and *Rhizobium sp* IRBG 74 stimulated early rice growth, increased grain and straw yields at maturity.

2.1.4 AZORHIZOBIUM : POTENTIAL AS BIOFERTILIZER

Azorhizobium belongs to family Rhizobiaceae that includes *Mesorhizobium*, *Sinorhizobium*, *Rhizobium*, *Allorhizobium* and *Bradyrhizobium* collectively called Rhizobia. These bacteria form peculiar symbiotic structures (nodules) on the roots of legume hosts. Generally, nodules are formed on roots but in certain plants stem nodulation is also reported. *Sesbania* and *Aeschynomene* show stem nodulation. Stem nodulating rhizobia include both fast (3-4 hr generation time) and slow growers (10 hr generation time) (Balasubramani and Kanmaiyan, 1990). The *Aeschynomene* nodulating rhizobia belong to slow growing group (Caroll, 1934). Johnson and Allen (1952) concluded that rhizobia of most *Sesbania* sp. belong to fast growing group and cross inoculation studies confirmed that *Sesbania* rhizobia bear a close relationship to cowpea (Johnson and Allen 1952 b).

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 .The root nodulating strain of *Sesbania* belong to genus *Rhizobium* while root and stem nodulating strain ORS571 has been shown as *Rhodopseudomonas palustris* (Jarvis *et al*, 1986). Cross inoculation of *Rhizobium* ORS571 of *S.rostrata* with *S. bispinosa* and *S.pachycarpa* caused ineffective nodulation in latter two *Sesbania* sp. (Dreyfus and Dommergues, 1981). None of *Aeschynomene* sp. rhizobia nodulate *S. rostrata* or vice-versa (Eaglesham and Szaley, 1983).

Thus *S.rostrata* was found to be associated with two rhizobial strains, ORS571 strain that fixes atmospheric nitrogen in culture media, grows at expensive of nitrogen and nodulates both root and stem of *S. rostrata* (*Azorhizobium caulinodans*) and other that nodulates only roots and does not fixes nitrogen in defined media (*Rhizobium*) (Dreyfus *et al*, 1985). The exact taxonomic hierarchy was assigned to the former one by numerical analysis of phenotypic characters, comparative protein gel eletrophoresis, DNA-DNA and DNA-rRNA-hybridization of root and stem nodulating strains of *S.rostrata*, *Rhizobium* and *Bradyrhizobium* (Dreyfus *et al*, 1988). The results led to the proposal of a new genus viz *Azorhizobium* containing a single species *A. caulinodans* as it constituted a separate r-RNA sub-branch on *R. palustris* r-RNA branch different from *Rhizobium* and *Bradyrhizobium* .

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.rostrata* showed G+C content ranging from 65-68%. Molecular genetics of nitrogen fixation of *A.caulinodans* ORS571 was studied by de Bruijn *et al* (1988) and Fischer (1994). 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).

MORPHOLOGY

The morphology and biochemical reaction of *A.caulinodans* was studied by Kalidurai and Kannaiyan (1988). Balasubramani and Kannaiyan (1992) reported in vitro screening media for stem and root azorhizobial isolates of *S.rostrata*. The cells of *A. caulinodans* were gram negative, small motile rods, 0-5-0.6 μ in width and 1.5-2.5 μ in length (Dreyfus *et al*, 1988). The colonies on Yeast Extract Mannitol agar appear circular, convex, creamy, viscid, translucent, butyrous, gummy and mucoid due to production of extracellular lipopolysaccharides. The bacteria were found to be able to utilise glucose, mannitol, sucrose, maltose and lactose (Neal and Walker, 1935).

2.1.5.2 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. A limited number of rhizobia of Genera *Bradyrhizobium* show free-living nitrogenase activity (Ramasamy and Bal, 1986). But nitrogen fixed by *Bradyrhizobium* strain CB756 under free-living conditions was 10 times lower than that by *A. caulinodans* ORS571 (Alazard, 1990). Kalidurai and Kannaiyan (1988, 1991) have shown the nitrogen fixing capacity of *A.caulinodans* in presence of fertilizer nitrogen at 2.5 ppm and also that *in vitro* nitrogenase activity was recorded relatively more in arabinose and mannitol followed by arabinose and sucrose in semi-solid agar medium. Thus nitrogenase activity of rhizobial strain on defined media depends on provision of suitable carbon source (Keister, 1975, Pagan *et al*, 1975).

Dreyfus *et al* (1988) reported growth of *A. caulinodans* on nitrogen under microaerobic conditions and nitrogenase activity around 30 nmoles of C_2H_4 mg^{-1} protein min^{-1} . Free-living *Rhizobium* *sp.* fixes nitrogen in liquid medium under low oxygen tension (Tjepkema and Evans, 1975). *Azorhizobium* was reported to have higher tolerance to oxygen (up to 9 μ moles) by Gebhardt *et al* (1984). A similar report by Urban *et al* (1986) suggested nitrogen fixation by strain ORS571 at high oxygen concentration (3%) and temperature (37°C), without differentiation in to bacteroids (Kitts and Ludwig, 1994). Recently Gough *et al* (1997) reported oxygen tolerance upto 12 μ Moles of dissolved oxygen.

2.1.5.3 NODULATION BY *A. caulinodans*

Trinick (1973, 1988) first reported rhizobial symbiosis with a non-legumes i.e *Parasponia-Rhizobium*, which provided a thrust for scientists with interest in BNF. Thus many of widely accepted dogmas for normal symbioses such as root hair infection and infection thread formation, are not universal (Sprent and deFaria, 1989). Wound infections are well-established paths for nodule initiation in legumes (Sprent and deFaria, 1989) so that idea of a fixed obligatory procedure cannot be sustained although such schemes provide helpful conceptual models for study.

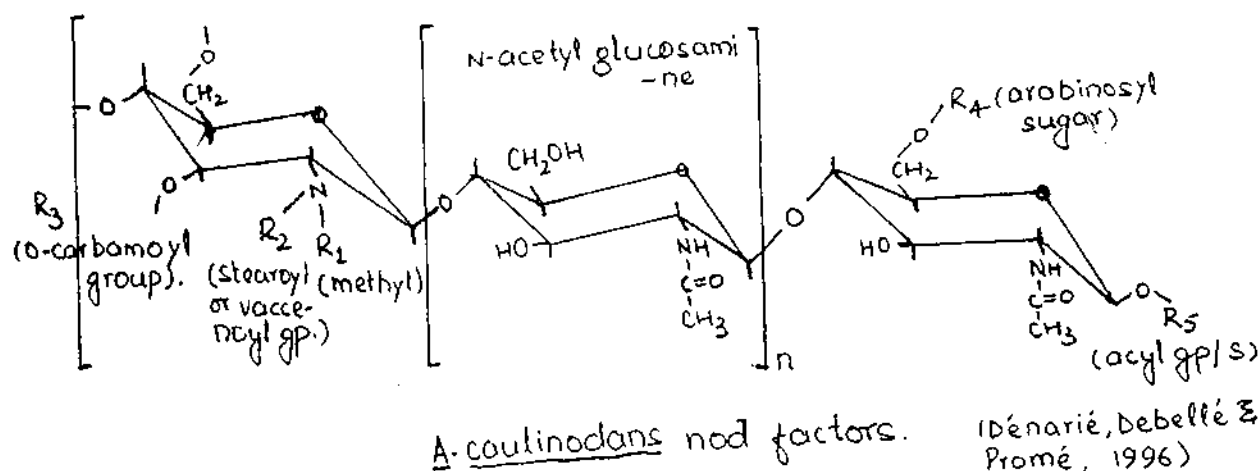
Parasponia rhizobia *Bradyrhizobium* cells initially enter *Parasponia* roots at localised sites of plant cell division having disrupted epidermis (Bender *et al*, 1987) and later form infection thread. Infection threads were reported absent during lupin nodule formation upon infection by *R. lupini* (Haack, 1961). The

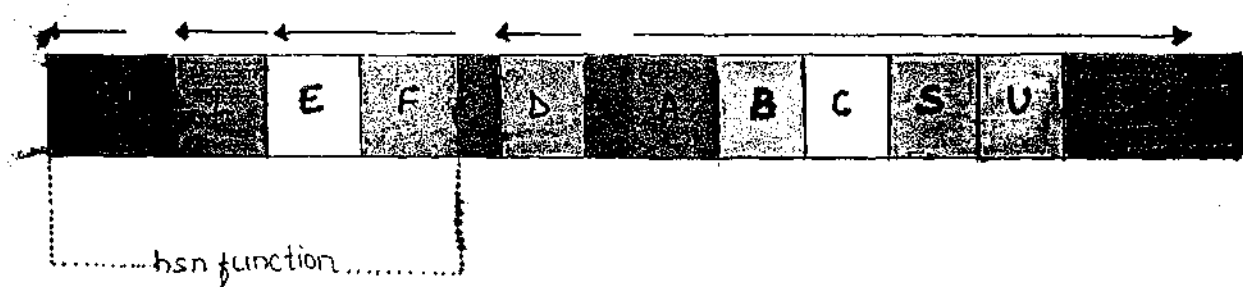
physiological comparison of root and stem nodule of *Aeschynomene*, *S. cabra* and *S.rostrata* was studied by Hungria *et al* (1992).

Induction of nodule formation by rhizobia involves signal compound exchange with in two symbionts, The signal exchange triggers cascade of reaction which activates nod operon consisting genes that code for synthesis of specific lipochitooligosaccharides 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).

Azorhizobial nod factor consists of lipo-chitooligosaccharide chain as core which contains a trimer to pentamer of β , 1-4-linked N-acetyl glucosamine (GlcNAC) residues and a fattyacyl moiety that replaces GlcNAC 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 stearoyl chain. On 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 (Mergaert *et al*, 1995; Goethals *et al*, 1992). Tomekpe *et al* (1996) reported that the greater infectivity of *A.caulinodans* ORS571 was due to more efficient production of mitogenic nod factors at stem located infection sites. The sequence surrounding the *A.caulinodans* regulatory nod D gene has also been analyzed (Geelen *et al*, 1995).

Nod factors, which are rhizobial signal molecules, induce specific gene expression in the cognate host plant (Denarie and Cullimore, 1993). The plant genes specifically expressed during the formation and functioning of nodules are referred as the nodulin genes (Van Kammen, 1984). Nodulin genes belong to two most prominent classes called 'Early' and 'Late' nodulin genes (Nap and Bisseling, 1990) and are clearly activated in different ways (deBruijn and Schell, 1992). Purified nod factors have been found to activate early nodulin genes such as Enod 5 and Enod 12 in the host plant. (Horvath *et al*, 1993) supporting role of Nod factors as essential signal molecule in nodule ontogeny. In addition to lipooligosaccharide signal molecules, other metabolites as hormones (deBruijn *et al*, 1992; Hirsch, 1992) and compounds as N-acetyl glutamic acid (Hollingsworth *et al*, 1989) and diglycosyl diacylglycerol (Orgambide *et al*, 1994). The latter compounds were found to elicit root hair deformation and have mitogenic activity on host root cells. (Hollingsworth *et al*, 1989; Orgambide *et al*, 1994). Sabry *et al* (1997) showed that wheat grown in pots and inoculated repeatedly with *A. caulinodans* colonized root tissues at point of emergence of lateral roots and appeared to contribute significant amount of nitrogen fixation to plant.





Nod Operon

nod F: Acyl-carrier protein

nod FE: involved in conferring host range

nod L: involved in root –hair curling

Nodbox: reiterated DNA sequence of 26-28 bases flanked by repetitive DNA sequences

nod A: 21.8 Kda protein located in cytosol adds acyl chain

nod B: deacetylase, removes N-acetyl group from non-reducing ends.

nod C: 44 Kda, integral membrane protein produces chitooligosaccharide.

nod D: regulatory gene. Nod D interact with plant stimulatory compounds and nod box to start transcription

nod S: Nods is an Ado Met-dependent methyl transferase involved in the Nod factor methylation.

nod U: involved in carbamoylation

nod Z IJ : involved in arabinosylation of Nod factors.

2.1.6 INDUCED NODULATION IN CEREALS

2.1.6.1 USE OF PHYTOHORMONES

Various genetic and non-genetic approaches are being used to induce nodule-like structures on roots of rice and other cereals. Application of 2,4-D on roots of non-leguminous plants causes induction of nodule-like structures called 'paranodules' (Tchan and Kennedy, 1989; Ridge *et al* 1992; Khush and Bennett, 1992), by rhizobia and free-living diazotrophs suggest a potential vehicle to achieve nitrogen fixation in cereals (Bender *et.al*, 1990; Tchan *et.al*, 1991). Infact nitrogen fixation by *Azospirillum brasilense* and *A. caulinodans* in paranodules induced on wheat roots has been reported (Chen, 1993; Chen *et al*, 1992). Christiansen-Weniger and Vanderleyden (1994) have shown that ammonia-excreting *Azospirillum* can become established intercellularly in 2,4-D induced paranodules of maize. Researchers are interested in obtaining an effective nitrogen fixing system for non-legumes field crops (Kannaiyan, 1997). Paranodulation has been reported along the primary and secondary roots on 2,4-D and *A. caulinodans* treatment of rice seedlings and it was found that endophyte established inside rice paranodules and in root tissues, around tumour bases suggesting root tumour induction as a suitable method of establishing endophytes in roots of graminaceous crops (Christiansen–Weniger, 1996 and Amutha and Kannaiyan, 1999).

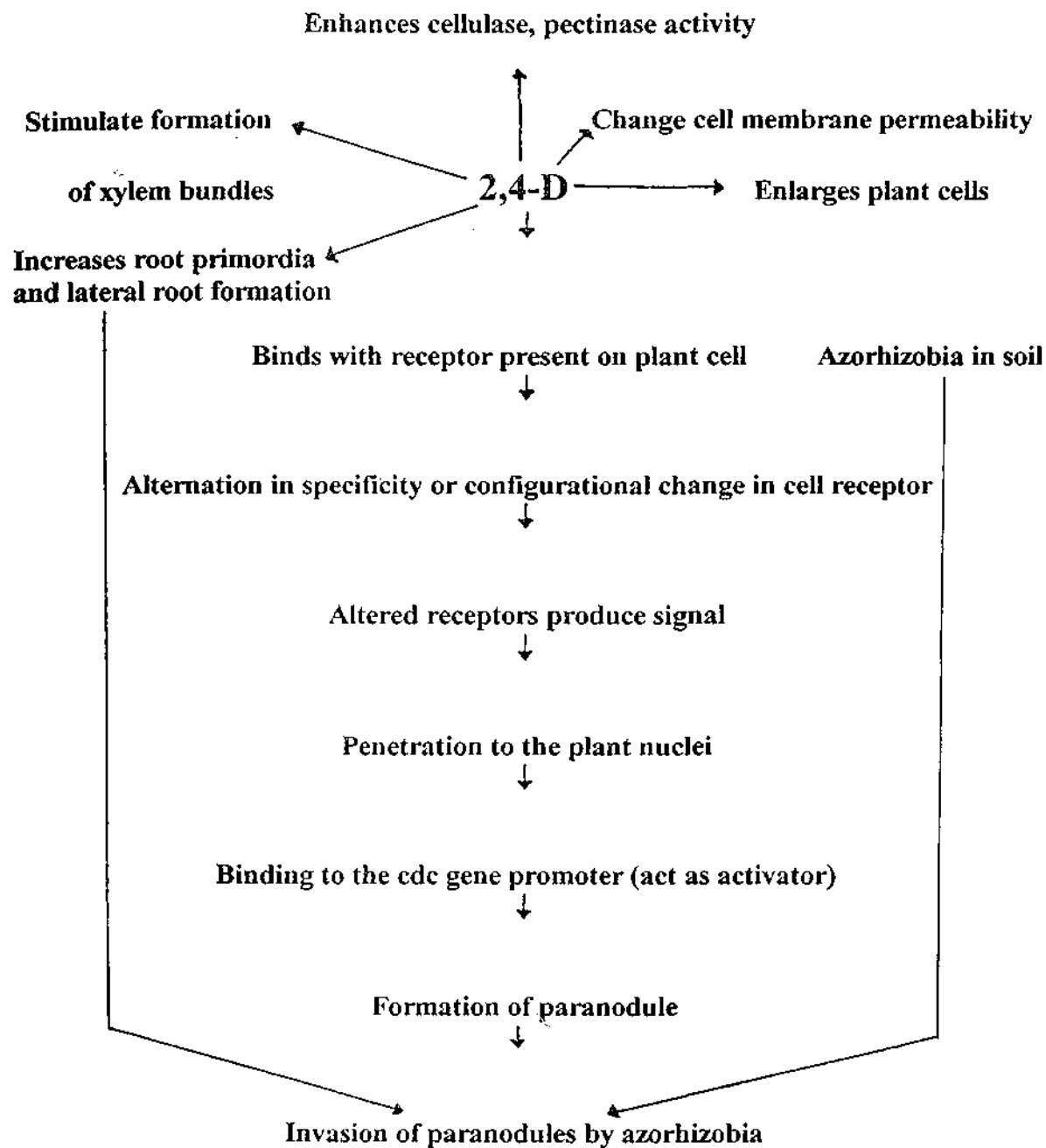
Nie (1983,1989) reported that 2,4-D induced paranodules were inhabited by diazotrophs. The *Parasponia* and the *Aeschynomene rhizobium* produced nodule-like structures on emerging lateral roots of rice, wheat and maize without

any enzyme treatment (Cocking *et al*, 1992). At recommended concentration of 2,4-D viz. 0.5, 1.5 and 3.0 ppm nodule-like structures were formed on rice, maize and sorghum roots due to *A.caulinodans* inoculation (Chandrasekar and Kannaiyan, 1995).

Physiologically, 2-4-D in lower concentration causes stimulation of plant growth and development, enhancement in sugar, starch and stored carbohydrate consumption. Mechanistically, 2-4-D has 100 times stronger activity than IAA. It works as polyploid inducer of plants causing plant cell enlargement and thickens roots. It also affect cell agglutination, affects plant nuclei and cell membrane permeability. It stimulates formation of Xylem bundles, root primordia lateral roots and paranodules formation (Modified lateral roots). It also enhances activities of enzymes as cellulase, pectinase in cells (Nie 1993). Rolfe and McIver (1994) reported induction of MRO's, which resulted from meristem induction on exogenous 2,4-D application to rice and wheat plants. 2,4-D treatments induced nodule-like structures in non- nodulating soybean T- 201 in presence of rhizobia that developed infected zone and exhibited nitrogen-fixing activity. Rice paranodules colonization and nitrogen fixation by *A. caulinodans* ORS 571 in sterile media was reported by Shizhen and Dongwei (1994) and Zhiguo *et al* (1994).

2.1.6.1(a) POSSIBLE MOLECULAR MECHANISM

2,4-D is endowed with ability to overcome the barrier of root cell walls .The exact effect of 2,4-D on agglutination, recognition or elimination of receptors on cell wall is not known. 2,4-D molecule itself is capable of inducing pseudonodule



FUNCTIONS AFFECTED BY 2,4-D FOR FORMATION OF PARANODULES

formation i.e. the paranodules primordia. 2,4-D switches on cell division genes by interacting with them. It may combine with receptors present on plant cell causing configurational changes in them. These changed receptors may move inside or may send secondary signals that penetrate to the nucleus of plant cell and bind with cell division cycle genes. Activation of cell division leads to formation of paranodules primordium, which provides optimum environment for bacteria to penetrate into cells and form paranodules (Nie, 1993). Pan *et al.* (1998) observed that *R.sesbaniae* and *A.caulinodans* inoculation on 2,4-D treated rice roots gave maximum nitrogenase activity of 77.6 nmole plant⁻¹ day⁻¹ with nodulation rate of 20.5% to 100%. This association was found to promote growth in rice.

Other phytohormones such as indole-3-acetic acid, Napthalene 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). Amutha and Kannaiyan (1999) reported that inoculation of rice with *A.caulinodans* along with growth hormones, 2-4-D or NAA at low concentrations induced rootlets, paranodules and nitrogenase activity. Increase in concentration of 2,4-D, NAA and Kinetin upto 7.0 ppm along with *A.caulinodans* inoculation increased the nodular structures and nitrogenase activity in rice, maize, sorhum, cumbu and ragi (Kannaiyan, 1998). Reddy *et al* (1997) reported that IAA produced by rhizobia seems to promote formation of 'thick short lateral root' (TSLR) in rice. Dehio and de Bruijn (1992) reported that cytokinin plays an important role in signaling and is required for nodulation since it determines the early nodulin gene Enod2 expression and promote nodulation deficient rhizobia (lacking common nod genes) to induce

nodulation in plants. Except Enod2 other early nodulin genes such as Enod124 and Enod40 have also been reported to be induced by cytokinins. (Hirsch and Fang, 1994; Bauer *et al*, 1996).

2.1.7. USE OF HYDROLYTIC ENZYMES

The first classical and landmark experiment regarding induction of paranodules in rice by rhizobia by using enzymatic removal of cell wall at root hair tips was done by Al-Mallah *et al* (1989a). The removal of apical cells wall by mixture of cellulase and pectinase enzymes expose plasma membrane and abolish specificity and recognition signaling steps. The bacteria then gain entry between two cell membranes due to degradation of middle lamella between root epidermal cells (Cocking *et al*, 1994). Coupling of cell wall degradation along with Polyethylene glycol (PEG) treatment promotes better bacterial encroachment and hence paranodulation in non-legumes. *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; Kennedy and Tchan, 1992). The cell wall hydrolysing enzyme treatment enabled normally excluded *R.loti* to infect clover (*Trifolium repens*) plants and form nitrogen-fixing nodules. Enzyme treated roots of rice, maize and oilseed followed by rhizobial inoculation caused nodulation in these non-legumes at a low frequency. Omission of enzyme treatment resulted in no such structure formation (Hu *et al*, 1993; Cocking *et al*, 1993). Hu Xiaojia *et al* (1993) and Cao and Li (1993) reported that use of a pectinolytic bacterium along with Rhizobial inoculation led to paranodulation in non-legumes. Bacteria in nodule structures

were mostly located between cell layers with some infection of cells that usually appear to lack well-defined cytoplasm. A synergistic interaction between *R. loti* and *Bradyrhizobium* produced greater rate of nodulation than that reported by Rhizobia alone (Kennedy and Tchan, 1992). Invasion occurred both within and between the nodule cells. Nodules, which developed on oilseed rape, reduced the C₂H₄ per unit fresh weight very feebly, at less than 0.1% of that seen typically in legume nodules. Some reports were given about induction of nodule-like structures and invasion of rice, maize and wheat seedling roots by *Parasponia* and *Aeschynomene* rhizobia either spontaneously or after treatment of roots with cell wall degrading enzymes. (Al-Mallah *et al*, 1989b; Cocking *et al*, 1990).

2.1.8 USE OF SIGNAL COMPOUNDS AND PLANT-MICROBE INTERACTION:

Various plant derived compounds were isolated in the root exudates that were identified as members of huge classes of secondary metabolites involved in several plant-microbe signaling interaction modules (Charrier *et al*, 1998) such as allelopathy plant growth regulation, defense reactions involved in plant-pathogenic interaction (Dixon *et al*, 1983). These secondary compounds or phenolics possess either activating or antagonizing properties (Firmin *et al*, 1986; Djordjevic *et al*, 1987). The root exudates act as important pre-invasive factors affecting root colonization by bacteria (Chatel and Parker, 1973) inducers act as chemoattractants (Harwood *et al*, 1984) and this chemotaxis requires prior exposure of microbes to compounds suggesting sensing and signal transduction induction in microorganisms (Parker *et al*, 1985).

Induction was found to depend on the structure of the phenolic compound, acetosyringone showing the greatest activity and found to be of effective inducers (Bolton *et al*, 1986). Differences in internal and external exduates contents were reported in some plants, which may be attributed to synthesis or the export of these compounds (Yelton *et al*, 1987). *Bradyrhizobium* and *Rhizobium* get attached by phenolics that act as potential nutrients (Peters and Verma, 1990) but at very-very low concentrations by compounds that may not be of nutritional value such as flavonoids (Kape *et al*, 1991).

Flavonoids are phenolics found systemically in a wide variety of plants with exception of isoflavonoids, which are almost exclusively found in legumes. Flavonoids are synthesised by host plant and secreted into rhizosphere where they act as molecular signals that activate bacterial infection (Long, 1989; Phillips, 1992). Exudation of an appropriate flavonoid signal from plants is first step towards root nodule formation and symbiotic nitrogen fixation (Hungria *et al*, 1992; Kapulnik *et al*, 1987). Flavonoids are produced both by legumes as well as non-legumes. Flavonoids secreted into rhizosphere of legume plants induce expression of a variety of genes in plant-associated bacteria such as *Rhizobium* (Mulligan and Long, 1985; Peters and Verma; 1990).

The target of these compounds is nodD gene product i.e. NodD protein that act as an 'environmental sieve' by sensing relative concentrations of stimulatory and antagonizing compounds released by a particular host legume. (Fischer and Long, 1992). Four families of flavonoids (flavones, flavonols, flavonones and isoflavones) have been shown to process nod gene inducing

activity (Denarie *et al*, 1992) nod D gene product get converted to active form by phenylpropanoids such as 7,4'-dihydroxyflavone (clovers), luteolin (alfalfa), apigenin 7-O glucoside and eriodictyl (Pea) at low concentration. These hydroxy and isoflavones are excreted predominantly from zone of emerging root hairs and production is developmentally regulated (Zuanazzi *et al*, 1998). Specific Nod-D-flavone interactions promote expression of nod ABC and nod FE operons and other inducible nod genes such as nod H or nod M.

The nod D gene product of *Rhizobium* strain NGR234 gets activated by simple phenolic compounds vanillin and isovanilline present in wheat root extracts (Le Strange *et al*, 1990). Isoflavone Daidzein isolated from soyabean roots induces nod genes of *R. fredii* and *Bradyrhizobium japonicum* (Sadowsky *et al*, 1988; Kosslak *et al*, 1987). Alfalfa leaves also contain daidzein (Guggolz *et al*, 1961) while *R. meliloti* inoculation to alfalfa roots causes exudation of formononetin-7-O' and medicarpin, which induces, nod gene expression (Phillips *et al*, 1994). Alfalfa also excretes hesperitin (Firmin *et al* 1986) production of these is nitrogen regulated (Coronado *et al*, 1995).

The plant signals, including flavonoids cause selective stimulation of specific microbial communities in rhizosphere (Philips and Streit, 1996). Moreover root exduates differentially produce or contain specific stimulatory signals and thus signal molecule class and concentration differ in legumes and non-legumes (Redmond *et al*, 1986). Bender (1988) reported occurrence of very common signals in diverse groups of legumes, non-legumes *Parasponia*, *Trema*, *Casuarina* and cereals, rice, maize and wheat plant extracts that induced nod D

gene of NGR 234 strain. A single plant can produce several different classes of phenolics or flavanoids (Hungria *et al*, 1992). Flavonoids or signal compounds may include glycosides as kaempferol in *Vicia hirsuta*, TCA intermediates, amino acids or sugars as in chickpea and aldonic acid as in *Lupinus albus* root extracts that can induce nod genes of various Rhizobia (Recourt *et al*, 1992; Kush and Dadarwal, 1981; Gagnon and Ibrahim, 1998) Flavonoids released as aglycones or glycosidic conjugates possess lower activity and more solubility (Maxwell and Phillips, 1990) Cereals such as wheat, produces vanillin, syringaldehyde, p-hydroxy benzaldehyde, apigenin and triclin (Hartley and Keene 1984; Feng *et al*, 1988; Anderson 1932). Usami *et al* (1988) identified vir gene activating compounds in extracts of oats and wheat seeds. Flavonoids such as vanillin and isovanillin were identified in wheat seedling extracts which act as capable inducers of nod operon of Rhizobium (LeStrange *et al*, 1990).

Flavonoids are produced and induced too in a site-specific manners in nodule and accumulate in the cytoplasmic membrane of the cortical cells after rhizobial infection. Flavonoids are then released in the rhizosphere as most of them or their derivative cell identified to be water-soluble. Later flavonoids reach rhizobial cell membrane; the region where threshold amount of Nod D protein cell membrane; the region where threshold amount of Nod D protein is localised (Mathesius *et al*, 1998); Recourt *et al*, 1989; Schlaman *et al*, 1989) and Nod D activation by flavonoids switches on the transcription of nod D-dependent nod genes for production of chitoligosaccharide or nod factor. Binding of nod D protein to nod box is enhanced by in *A.caulinodans* (Goethals *et al*, 1992). Thus

flavonoids play important role in altering hormonal balance leading to cortical cell division. This unsheaths an entirely new arena regarding the role of flavonoids in nodule formation.

Naringenin, a flavonone most efficiently induces the *A.caulinodans* ORS571 nod genes and this induction leads to inter cellular crack entry infection of *S.rostata* (Goethals *et al*, 1990). The natural inducer in *Sesbania rostrata* seedling exduates was identified to be 7,3-dihydroxy flavanone, which was found to be structually related to naringenin (Messens *et al*, 1991). Use of lacz marker gene showed that naringenin at 10^{-4} to 10^{-5} M concentration stimulated significantly the colonization of lateral root cracks and intercellular colonization of wheat roots by inoculated azorhizobia (Gough *et al*, 1996; Webster *et al*, 1997). Senthil Kumar (2000) reported induction of nodule-like structures in rice and maize when inoculated with *A.caulinodans* along with enzyme mixture and naringenin at 3.0 ppm. This also enhanced rhizoplane population in pot cultures studies. Other flavonoids such as daidzein, luteolin, myricetin, quercetin etc showed no effect (Gough *et al*, 1997) as LRC colonization but they stimulated lateral root formation. Naringenin did not stimulate *R. meliloti* to colonize wheat roots suggesting its specificity towards *A. caulinodans* strain ORS571. Thus flavonoids are a part of regulator system for *A. caulinodans* colonization (Kannaiyan, 1998).

NodC and nod D mutants of ORS571 were able to colonize wheat LRC's as well as wild type strains (Webster *et al*, 1997; Gough *et al*, 1997; Goethals *et al*, 1990). Thus naringenin stimulation of such mutants suggests that it acts as a

signal and not as a substrate. Nod D binding to nod box of nod operon of *A. caulinodans* is enhanced by naringenin. Rice, among cereals has been taken as the model monocot for studying the potential for extension of BNF to non-legumes (Ladha and Reddy, 1995). Primary constraint regarding diazotrophic colonization in rice is the lack of appropriate nod gene activators in rice root exduates or rice may excrete antagonistic compounds capable of inhibiting nod gene expression (Reddy *et al*, 1997). Generally, coumarins and isoflavones antagonize nod gene stimulation, are released from region between small emerging root hair (SERH) zone and root tip and elsewhere on root. Activation of nod genes determined by stimulator to inhibitor ratio as variation in S:I ratio over developing root determines site of nodule initiation. Fluctuations in concentrations of theses compounds in plant particularly legumes affect nod gene expression in infection thread. An excess of antagonizing compounds may cause infection thread to cease growing because bacteria are not able to stimulate plant to continue either synthesis of infection thread cell wall or cortical cell division (Rolfe and Gresshof, 1988).

2.1.9 MOLECULAR GENETIC TECHNIQUES OF CEREAL NODULATION

With the advent of several new discoveries in molecular genetics it has become easier to dissect various metabolic processes. Extension of legumes-rhizobial symbiosis to non-legumes is a luring subject due to its enormous economical and environmental value. Plazinski *et al* (1985) performed the successful transfer of genes associated with root hair curling (hacgenes) from *R. trifolii* into a derivative *R. trifolii*, on plasmids pKT290. Root hair curling character

was expressed in rice and maize seedling upon rhizobial infection hac genes were also transferred from *R. meliloti* into *Azospirillum brasilense* on plasmid pVK-100.

Rolfe and Bender (1990) described further developments to evolve rhizobia for non-legume nodulation. They constructed a strain of *Rhizobium* reconstructed from one capable of nodulating *Parasponia* with a nod D allele inserted in genome was used to inoculate rice seedlings, occasionally resulted in nodule-like structures on roots. Thus genetically transformed rhizobia seem to be an interesting alternative approach for nodulation in rice, maize and wheat (Kennedy and Tchan, 1992; Okon, 1985).

Development of genetic tools based on application of lacZ and gus A fusions with genes associated with nitrogen fixation can assist effective expression patterns in associative systems of cereals. (Kennedy *et al*, 1997) Jing *et al* (1990b) have described formation of root nodules using a mutant of *Rhizobium sesbania* (*A. caulinodans*). The structure of these nodules and their formation was claimed to be similar to those of legumes nodules such as soybean, including the presence of leghaemoglobin. Incorporation of genes coding for protein degrading enzymes in native rhizobia by genetic manipulation could help or facilitate root invasion (Okon, 1985).

Several techniques regarding genetic transformation of non-legumes/ cereal host were also worked upon. Bogusz *et al*. (1980) transferred *Parasponia* leghemoglobin gene in *Tremia tomentosa* and found by southern and western blotting that it get transcribed in *Tremia* roots in a organ-specific manner.

Similarly Guo *et al.* (1998) and Wei *et al.* (1998) obtained transgenic rice plants transformed by addition of *Parasponia* leghaemoglobin gene. They observed organ-specific expression of transferred gene in transgenic rice. The transformation was performed by pollen tube and gene gun technique on rice florets. Ela *et al.* (1982) described an approach of breeding corn naturally inhibit rhizosphere and also observed nitrogenase activity in corn. Dixon *et al.* (1997) reported formation of transgenic tobacco and *chlamydomonas* by incorporation of *nif* genes and their expression in chloroplasts. Chloroplasts have the total metabolic machinery resembling prokaryotic origin. The transformed chloroplasts in dark period could synthesize nitrogenase after *nif* gene attachment.

Certain amount of work has been done on already existing rudimentary universal gene shared by both legume and non-legumes. Stacey *et al.* (1997) observed that chitin oligosaccharide released by fungal pathogens induced a plant defense reaction in rice so it could be hypothesized that rice, legumes and other plants may possess members of an evolutionarily role in chitin binding or reception and subsequent signal transduction cascades. They have also observed another chitin-binding receptor in legumes that has greater specificity for lipochitin nodulation signals.

2.1.10 SITE OF BACTERIAL COLONIZATION IN CEREALS

Endophytic establishment of certain bacteria in non-legumes does occur in cortical tissue and xylem. Xylem colonization has been reported in alfalfa by non-rhizobial endophyte (Gagne *et al.*, 1987). Similarly, xylem colonization in

several plants by bacteria has been reported without disease symptoms (Hallman *et al*, 1997; Bell *et al*, 1995).

Xylem vessels consist of basically sclerenchymatous tissue, which provide mechanical support, and structural or physiological robustness to plant (Canny, 1998). Moreover, they can provide low pO₂ conditions necessary for expression of nitrogenase enzyme and also can provide the metabolites exchange needed for survival of endophyte (James *et al*, 1994; Rolfe *et al*, 1997). Such endophytes remain in xylem without impairing transpiration (Van Alfen, 1982), while on the contrary benefit plant by enhancing shoot growth (Kloepper *et al*, 1992) or by contributing towards increased BNF as by *Acetobacter diazotrophicus* and *Herbaspirillum* sp. in Sugarcane (Boddey *et al*, 1995). *Acetobacter* and *Azoarcus* spp. Colonizes xylem vessels of sugarcane (Hurek *et al*, 1994) and rice (Englehard *et al*, 2000). Englehard *et al* (2000) also reported that number and diversity of diazotrophic endophytes associated with rice (modern) was higher than its putative wild rice. *Herbaspirillum* colonizes vascular tissue of Sorghum leaves (James *et al*, 1997).

Xylem colonization basically occurs in plants where bacteria enter roots by 'crack entry' as in certain legumes as *Arachis* spp. and *Sesbania rostrata* and also non-legumes *Parasponia* (Boivin *et al*, 1997; Ndoye *et al*, 1994). Bacterial invasion in such cases occurs through natural fissures created by epidermal erosion during emergence of the lateral root /rootlets. Bacteria colonize both nodules and xylem tissue as exhibited by microscopic studies of lacZ reporter gene containing *A. caulinodans* ORS571 (O'Callaghan *et al*, 1997). Prayitno *et al*

(1999) reported that *R. leguminosarum* bv *trifolii* preferentially colonized rice seedlings surfaces in clumps. This occurred along grooves on rice root also observed to colonize intercellularly in lateral roots formed on main roots near the culm region of seedlings. Recently Chaintreuil *et al* (2000) reported presence of *Bradyrhizobium* strain ORS278 in roots of *Oryza breviligulata*.

Cereal colonization has been widely studied in wheat and rice (Cocking *et al*, 1995). Wheat has been reported to be xylem colonized by azorhizobia, and by *Pantoea agglomerans*. Chen *et al* (1992) observed nitrogen fixation in wheat paranodules induced by *Azorhizobium* bacteria. Microscopic studies revealed that azorhizobia invaded intercellularly in cortical cells, xylem and root meristem. Youssef *et al* (1998) also observed similar phenomena.

Xueqin and YuXiang (1998) and Webster *et al* (1997) reported bacterial colonization of rice, barely and wheat roots. The former group reported appearance of protrusions on roots of rice seedlings IR-36 after rhizobial inoculation. Section under TEM indicated that rhizobia existed densely in intercellular space and cell respectively. The infected cell showed its coarse structure and the nuclear membrane, rhizobia appeared naked without any surrounding membrane. Though no nitrogenase activity was observed but the inoculated rice plants grew much better than the control plants. Christiansen-Weniger (1996) reported *A. caulinodans* endophytic establishment in rice treated with auxin.

Webster *et al* (1997) showed *A. caulinodans* ORS571 invasion in rice treated with naringenin via lateral root cracks and observed large intercellular

pockets of bacteria in cortex. Scanning electron micrographs confirmed crack entry by Azorhizobia at the point of emergence of lateral roots where epidermis get ruptured as well as heavy bacterial colonization around lateral root emergence sites (Reddy *et al*, 1997). Recently, Gopalaswamy *et al* (2000) demonstrated xylem colonization of *Oryza sativa* roots by *A caulinodans* ORS 571 (pxLGDA) being stimulated by flavonoid naringenin.

Chapter-III

MATERIAL AND METHODS

The present investigation was carried out in Department of Microbiology at Punjab Agricultural University, Ludhiana during 1999-2002.

3.1 GENERAL

3.1 1.1 Materials

3.1 1.1 Glasswares

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

3.1 1.2 Chemicals

The chemicals for experiments were obtained from Himedia Laboratories Pvt.Ltd., Mumbai (India), Qualigens fine chemicals, Mumbai (India), Genei Pvt., Bangalore (India), and Sigma chemicals Co., St. Louis (USA).

3.1 1.3 Soil

The soil for pot experiments conducted was collected from fields (Microbiology Department) and B- block field (Plant breeding Department), PAU, Ludhiana. Collected soil was dried, powdered, sterilized and used for the experiment.

3.1.1.4 Seeds

Seeds of *Sesbania rostrata* were obtained from the Farm House, PAU, Ludhiana. While seeds of Rice (*Oryza sativa* cv. PR-115) were obtained from Department of Plant

Breeding, PAU, Ludhiana in *in vitro* and field experiments.

3.1.1.5 *Azorhizobium caulinodans* cultures

The *A. caulinodans* cultures used in the present study were obtained from Department of Microbiology, Punjab Agricultural University, Ludhiana. The reference cultures *A. caulinodans* ZB-SK-5 and *A. caulinodans* BB-SK-5 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 % (HgCl_2) mercuric chloride for 3 mins. and washed repeatedly with sterile autoclaved distill water. Further surface sterilization was done by treating nodules with 70% ethyl alcohol for 30 secs; followed by sterile water washings. Nodules were crushed with a glass rod sterilized by flaming, in sterile water. Dilutions of suspension were made and 0.1 ml of it was spread on Yeast Extract Mannitol Agar (YEMA) medium.

The plates were incubated in the laboratory at room temperature ($28 \pm 1^\circ\text{C}$) for 48 h. Distinct, creamy, slimy, viscid, butyrous and translucent colonies were selected that did not absorb the Congo red dye. These colonies were picked up and streaked repeatedly for purification on YEMA plates and finally subcultured and maintained in YEMA slants in the refrigerator.

Composition (g l^{-1}) (Allen, 1953)

Mannitol	10.0
Dipotassium	
Hydrogenphosphate	0.5
Magnesiumsulphate	0.1
Sodium chloride	0.1
Yeast Extract	0.5
pH	7.0
Agar	20.0
Congored dye	10.0 ml

(Congo red stock solution 0.25 g /100ml distilled water).

3.2.2 Characterization of isolates

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 culture was poured and then YEMA was poured. Petri plates were incubated at $28 \pm 1^\circ\text{C}$. The fast growing isolates formed colonies in 48 h while slow growers appeared after 3 days. The growth pattern and phenotypic characters were thus recorded later.

3.2 2.1 Ketolactose Test

Test isolates were grown on lactose medium in petriplates and incubated for 3-4 days at room temperature $28 \pm 1^{\circ}\text{C}$

Composition (gl^{-1}) (Bernaert and Deley, 1963)

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

Growth on petriplates was flooded with Benedict's reagent and the reaction was observed. The *Agrobacterium* colonies stained yellow, while *Rhizobium* colonies remained white, giving negative reaction for confirmation of *A.caulinodans*.

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

Reagent A

Sodium citrate	-	17.3
Copper sulphate	-	17.3
Anhydrous Sodium carbonate	-	10.0
Distill water	-	1000ml

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 ($28 \pm 1^\circ\text{C}$) produced a colour change in medium. This reaction was observed for the respective azorhizobial isolates.

Composition (gl^{-1})(Norris, 1965)

Mannitol	10.00
Potassium dihydrogen phosphate	0.80
Sodium chloride	0.20
Ferric chloride	0.01
Yeast Extract	0.40
Bromothymol blue Indicator (0.4%)	5.00 ml
pH	7.20
Agar	20.00

3.2 2.3 Growth on Hofer's Alkaline Broth

A loopful of isolates was inoculated in the Hofer's alkaline broth and growth observed after 48 h. Absence of growth indicates positive reaction for *A. caulinodans*.

Composition (gl^{-1}) (Hofer, 1935)

Mannitol	10.00
Potassium dihydrogen phosphate	0.20
Magnesium Sulphate	0.20

Yeast Extract	0.50
Calcium Carbonate	0.05
pH	11.00

3.2. 2.4 Nitrogenase activity of Isolates

Growing *A.caulinodans* isolates in yeast extract mannitol broth performed acetylene reduction assay. After 48h incubation, cotton plugs of vials were replaced by subaseals and 10 % (v/v) 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 120°C. The sample gas fed to column. One ml of 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 cells.

3.4.2.4.2 Estimation of Protein content (Lowery *et al*, 1951)

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

Reagents:

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

Solution B: 0.5 percent copper sulphate in 1 percent 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:

The *A. caulinodans* isolates were grown in YEM–broth for 48 h and known volume of it was centrifuged at 10,000 rpm for 30 mins. The cultures suspension (0.5) was taken in a test tube and volume made up to 1 ml with distilled water 5 ml of Sol. was added to each tube and allowed to stand for 10 min. Then 0.5 ml of solution D was added, mixed by shaking and incubated at 25 °C for 30 min. Blue colour appears the intensity of (OD) of which was measured in Bausch and Lomb Spectronic–20 at 525 nm using the blue filter against a blank. The standard solution was taken of Bovine serum albumin that contained 200 µg of protein per ml.

A standard curve was prepared of the OD Vs µg protein of standard BSA solution and protein content was calculated using it. 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 on different antibiotic to study the anti-biotic resistance pattern. Aqueous solutions of different antibiotics were made with varying concentrations. Ethanol works as solvent for Tetracycline,

methanol for Chloramphenicol while 1M NaOH for Nalidixic acid. The stock solutions of 10 mg/ml were prepared in respective solvents and were sterilized by filtration through 0.2µm disposable membrane filter assembly (Millipore-GX).

Preparation of Antibiotic testing media:

Three different final working concentration of 10 µg /ml, 20µg /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:

Ampicillin	- Distil water
Kanamycin	- Distil water
Neomycin	- Distil water
Tetracycline	- 50 % ethanol
Chloramphenicol	- Methanol
Nalidixic acid	- 1M NAOH

Tetracycline was used at final working concentrations of 10 and 20 µg /ml while others were used at all three above mentioned working concentrations.

3.2.3. In vitro technique for raising rice seedlings

3.2 3.1 Seed sterilization

Rice seeds of PR-115 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 80x2.5 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 (80x2.5 cm) having a double-M whatman filter paper no.1 wick. 30ml of nitrate free media was sterilized by autoclaving (Plate 1). 48 h old culture isolates were added (250 µl) into 30 ml nutrient media 7 days of seedling growth. Three chemicals viz. 2,4-D, NAA and Naringenin were added in 3 different concentrations after 2 days of culture inoculations.

Plate 1: Rice seedlings (cv PR-115) grown on plant nutrient medium under lab conditions



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 (Broughton and Dilworth, 1970)

Stock solutions (g/50 ml)

Solution I: $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$	- 14.705
Solution II: $\text{KH}_2\text{PO}_4 \cdot 3 \text{H}_2\text{O}$	- 6.805
Solution III: $\text{FeCl}_3 \cdot 3 \text{H}_2\text{O}$	- 0.18
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	- 6.165
K_2SO_4	- 4.350
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	- 0.016
Solution IV: H_3BO_3	- 0.012
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	- 0.014
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	- 0.005
$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	- 0.002
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	- 0.0024

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

3.2 3.7 Chemicals

Three different chemicals were given to seedling roots in 3 different concentrations.

- 2,4-D (synthetic auxin) : 1% stock solution made in 50 % alcohol.
3 concentrations viz, 0.5, 1.5 and 3.0 ppm were used as working concentrations.
- NAA (Naphthalene acetic acid) : 0.7% stock solution made in 0.5 N NaOH
3 working concentrations of 3.0 ppm 5.0 ppm and 7.0 ppm were used.
- Naringenin (flavonoid) : Stock solution of 10^{-3} M was prepared in 0.1 m NaOH
working concentrations viz. 10^{-5} , 10^{-6} and 10^{-7} M were used.
- Acetosyringone : Sodium/Potassium salt of Acetosyringone was dissolved in 80% alcohol and volume made upto 10 ml with distill water and 2 concentrations used viz. 266 ppm and 300 ppm.

3.2.4 Effect of inoculation of *A. caulinodans* isolates and chemicals on growth and paranodulation in rice

3.2.4.1 Effect of *A. caulinodans* isolates inoculation with 2,4-D on rice.

Pre-soaked rice seeds var. PR-115 were surface sterilized by treatment with 0.1% mercuric chloride for 5-6 min followed by washings with sterile water .70% ethanol was later used for 3 min to further surface sterilize the seeds. Repeated sterile water washings were given and seeds were placed on moist filter paper containing petriplates. Uncontaminated, sprouted seeds were transferred to hydroponic system as described above. One week later 250 μ l of 48 h old *A. caulinodans* cultures i.e. R₃, R₄ S₁, S₂ and Reference strain ZB-SK-5

were added to the tubes. After 2 days synthetic auxin 2,4-D (2,4-dichloro phenoxy acetic acid) was added according to following treatments.

T₁ – Control 1- No culture and no chemical

T₂ – Control 2 – Culture only

T₃ – Control 3- Chemical only

T₄ – *A. caulinodans* + 0.5 ppm 2,4-D

T₅ – *A. caulinodans* + 1.5 ppm 2,4-D

T₆ – *A. caulinodans* + 3.0 ppm 2,4-D

Following parameters were observed

- Root length – Root length was taken using scale, from the root-shoot junction to the tip of 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.
- Number and size (small, medium or large) of nodule- like structures appeared on rice roots were recorded.
- Nitrogenase activity - Rice roots were washed thoroughly with tap water to wash away the media. These were blotted to remove drenching water and placed in 60 ml vials and closed with the rubber stoppers. 10% (v/v) of air (6 ml) was taken out using a sterile disposable syringe and same amount

of acetylene was added/ injected. Vials were incubated for 1 h at room temperature .0.5 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 nm of C₂ H₄ produced per plant per 20 hour.

- Azorhizobial intensity:

A. caulinodans isolates were re-isolated from rice roots by procedure given by Vincent (1970). Fresh rice roots were washed with distill 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 4.2 Effect of inoculation of *A. caulinodans* isolates and NAA on rice.

Rice seeds PR-115 were soaked in distill water for overnight and surface sterilized by 0.1% mercuric chloride and 70% ethanol and then washed repeatedly with distill autoclaved water. Uncontaminated germinated seeds were transferred aseptically to hydroponic system .NAA (Napthalene acetic acid) was added as,

T₁ – Control 1- No culture and no chemical

T₂ – Control 2 – Culture only

C₂ R₃- R₃ root isolates

C₂ R₄- R₄ root isolates

C₂ S₁- S₂ Stem isolates

C₂ S₂- S₂ Stem isolates

C₂ Ref-Reference ZB-SK-5 culture

Control 3- Chemical only

C₃ C₁: 3.0 ppm NAA

C₃ C₂: 5.0 ppm NAA

C₃ C₃: 7.0 ppm NAA

T₄ – Culture + 3.0 ppm NAA

T₅ – Culture + 5.0 ppm NAA

T₆ – Culture + 7.0 ppm NAA

Following growth parameters were observed in this experiment: -

- Root length- Root length was taken using scale, from the root-shoot junction to the tip of 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 50°C for 48 h and weighed on an electronic balance.
- Lateral roots – Number of lateral roots emerged were counted.
- Number and size (small, medium or large) of nodule-like structures appeared on rice roots were recorded.
- Nitrogenase activity - Rice roots were washed thoroughly with tap water to wash away the media. These were blotted to remove drenching water and placed in 60 ml vials and closed with the rubber stoppers. 10% (v/v) of air (6 ml) was taken out using a sterile disposable syringe and same amount

of acetylene was added/ injected. Vials were incubated for 1 h at room temperature. 0.5 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 nm of C₂ H₄ produced per plant per 20 hour.

- Azorhizobial intensity:

A. caulinodans isolates were re-isolated from rice roots by procedure given by Vincent (1970). Fresh rice roots were washed with distilled water 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 4.3 Effect of inoculation of *A. caulinodans* isolates and Naringenin on Rice

Rice seeds PR-115 were surface sterilized by treatment with mercuric chloride (0.1%) and 70% ethanol and further washed with autoclaved distilled water. Germinated seeds were transferred to hydroponic system and Naringenin was added as

T₁ – Control 1- No culture and no chemical

T₂ – Control 2 – Culture only

T₃ – Control 3- Chemical only

T₄ – Culture +10⁻⁵ M Naringenin

T₅ – Culture +10⁻⁶ M Naringenin

T₆ – Culture +10⁻⁷ M Naringenin

Following parameters were studied

- Root length
- Shoot Length
- Number of lateral roots
- Fresh weight
- Dry weight
- Nitrogenase activity
- Azorhizobial intensity

3.2 4.4 Microtomic studies for inter and intracellular incorporation of *Azorhizobium* in root /rootlets of rice

Rice root system inoculated with *A. caulinodans* isolates was washed with tap water and then blot dried to remove water. Roots were cut 1cm long. Nodule-like structures formed on rice roots were also washed with tap water.

FIXATION:

Samples were fixed in Formalin–acetic acid–alcohol (FAA) solution for 24h.

FAA solution

Formalin (40%) - 10 ml

Alcohol (50%) - 85 ml

Glacial acetic acid - 5 ml

DEHYDRATION:

Fixed tissues were dehydrated by passing through series (graded) of ethyl alcohol. (10, 30, 50, 70, 90, 95, 100 percent) each solution was changed for 2 hours and two changes with dehydrated alcohol were done. Now, ethyl alcohol:

Xylene (3:1, 1:1, 1:3) treatment were given to tissue for 2 hours each. Material was then given 2 changes with Xylene (pure).

INFILTRATION:

Paraffin wax of 58–60°C was added to tubes containing pure Xylene at 60°C in oven. Paraffin wax was added in half amount and corks of tubes were opened to allow evaporation of Xylene. Two changes of molten paraffin in wax were done at every 4 h interval

EMBEDDING:

The tissue in pure paraffin wax were embedded in pure wax poured in two L-shaped structures placed on glass. Roots were placed suitably using a scalpel /needle. Small rectangular blocks of paraffin wax obtained on hardening of wax. The edges of blocks were trimmed with the help of a scalpel to obtain smooth surface for microtomy.

MICROTOMY:

The trimmed blocks were fitted on a rotary microtome and serial sections of 10µm thickness were cut. The ribbons having serial sections were mounted on glass slides using egg albumin.

Egg Albumin composition:

White of Hen's egg - 50 ml

Glycerine - 50 ml

Sodium salicylate - 1 g

Shake the white of an egg with few drops of acetic acid thoroughly for half an hour. Then add other constituents and shake for half an hour. Filter the contents

through thick layer of muslin cloth. The egg albumin is allowed to mature for 2-3 months.

Egg albumin was applied using brush on a pre-cleaned slide, as a thin film and paraffin ribbons were placed on the slides. The section were flooded with water on a hot plate so as to, expand the paraffin wax sections. The slides were cooled and excessive adhesive was wiped off with a filter paper around the edges of the ribbon.

DEWAXING:

Dewaxing was done by giving two changes of pure xylene for 30 minutes each. The section were hydrated by downward series of

Xylene + alcohol (3:1) - 15 min

Xylene + alcohol (1:1) - 15 min

Xylene + alcohol (1:3) - 15 min

Absolute alcohol - 10 min

Alcohol 90% - 10 min

Alcohol 70% - 10 min

Alcohol 50% - 10 min

Alcohol 30% - 10 min

Alcohol 10% - 10 min

STAINING:

The slides were brought to water and stained with (5%) safranin stain (5g safranin stain in 100ml distill water). Slides were immersed in stain for 7 min. Then the slides were washed with distill water and passed through upward series of alcohol and absolute alcohol rapidly.

The slides were then immersed in (0.5%). Fast green stain solution (0.5g) fast green in 95% alcohol) for 10 min. then slides were passed through an upward series of

Absolute alcohol - 10 min

Alcohol+ Xylene (3:1) -15 min

Alcohol+ Xylene (1:1) -15 min

Alcohol+ Xylene (1:3) -15 min

Pure Xylene I - 10 min

Pure Xylene II - 15 min

Finally sections were mounted using DPX mountant and studied under compound microscope for identification of sections in which *A. caulinodans* could be observed lying on cell surface or in between cells.

3.2 5. Effect of inoculation of *A. caulinodans* stem/root isolates on *Sesbania rostrata*

3.2 5.1 POT EXPERIMENT

The pot experiment was done in loamy sand soil in small pots. About 3 kg of soil was filled in each pot and on *Sesbania rostrata* seeds were used. Four to five plants were maintained in each pot. The seeds were treated with *A. caulinodans* (6 isolates: R₁, R₂, R₃, R₄, S₁, S₂, and ZB-SK-5 reference) cultures. The cultures were inoculated by mixing 48 h old isolates with seeds and charcoal powder. Seeds were rubbed with charcoal-culture mix so that they get properly coated with the inoculum. The treated seeds were shade-dried and then sown in pots. The pots were previously sprinkled with water and 255 mg of super phosphate was added on same day of date of sowing of seeds.

After 45 days, the nodulation number and mass, nitrogenase activity, biomass and competitiveness of inoculated *Azorhizobium* strains was recorded

1. Plant Height: The height was measured using a scale and height was taken from point of shoot–root junction to the meristem.
2. Root and shoot weight: The root and shoot were measured on a weight balance.
3. Plant biomass: The plants were carefully uprooted and the weighed on a weigh balance.
4. Nodulation number and mass: The root and stem nodules were detached and weight on electronic balance. The nodules were also dried at 60°C for 48 h and weighed on electronic balance.
5. Nitrogenase activity: The roots were cut at stem-root junction and attached soil was removed. The roots with intact nodules were incubated for one hour at room temperature with acetylene injected to 10 % volume of the vessel. The sample gas (1ml) was injected in reactivated Gas chromatograph (Nucon). The number of chart units moved were recorded and the nitrogenase activity was calculated according to formula,

1mole of ethylene at NTP= 22.4l

22.4ml will contain = 1m mole of C_2H_4

1 ml will contain = $1/22.4 = 44.6 \mu$ moles

1 ml of standard C_2H_4 moves = X chart units

X cu will have = 44.6μ moles

1 cu will have = $44.6 / X = Y \mu$ moles.

$$\text{Nitrogenase Activity} = \frac{\text{Vessel volume} \times \text{c.u of standard (k)} \times \text{c.u. of sample}}{\text{Incubation period}}$$

The nitrogenase activity was expressed as nM/plant.

The nitrogenase activity was measured as mentioned above. About 10 cm of the shoot length was cut at the region where maximum number of shoot nodules were present. The cut shoots were incubated with acetylene (10% volume of vessel) for 1 h and the nitrogenase activity was measured on a Nucon gas chromatograph. The nitrogenase activity was expressed as μmoles of ethylene per 10 cm of the shoot containing stem nodules.

6. Competitiveness of inoculated strains: The nodules from root and stem were re-isolated as described in section 3.2 1.1. The combinations of antibiotic solutions were added in YEMA media for the respective isolates. Three nodules were streaked on YEMA medium. Only those colonies, which have the same antibiotic spectra as that of the isolates (respective) grew on antibiotic, enriched media.

3.2 5.2 FIELD EXPERIMENT

The field experiment was performed in the Microbiology field. Twenty-four plot of size 1.10m x 2.00 m were prepared and ploughed before sowing to get rid of weeds. *Sesbania rostrata* seeds were rubbed with 48 h old culture isolates of *A.caulinodans* and reference strain ZB –SK-5 along with charcoal powder. The control seeds were not treated with culture. The seeds were sown and 40.7 gm of super phosphate was also added per plot according to recommended dose of

75 kg. Then sampling was done after 45 days after date of sowing to study following parameters.

1. Plant weight: The height was measured in centimeters using a scale and measured from root –shoot junction to the meristem of each plant.
2. Root and shoot weight: The weight was measured in grams with the help of a weight balance.
3. Plant biomass: Plants were carefully uprooted from field and fresh weight was taken .The plants were dried in an oven at 60 °C for 48 h and weighted on weigh balance.
4. Nodulation number and mass: The root and stem nodules were detached and weight on electronic balance. The nodules were also dried at 60°C for 48 h and weighed on electronic balance.
5. Nitrogenase activity: The roots with intact nodules were cut at the root-shoot junction and thoroughly washed of attached soil with tap water. The roots were blotted off to get rid of moisture. The root were placed in vessel and stopped with rubber cork 10% of the air of vessel was injected out with the help of a sterile syringe and same amount of acetylene was injected inside vessel. The roots were incubated for 1 hour at room temperature. 1 ml of sample gas was taken from each vessel and introduced in gas chromatograph. The column was also calibrated with standard ethylene gas. The numbers of chart units moved by indicator were counted and activity was expressed as nM of C₂H₄ per plant per hour.
6. Competitiveness of strains: The procedure was performed as described in section 3.2.5.1(6).

7. Yield: The amount of grains obtained were weighted on a weigh balance and expressed as grams of grain obtained per plot.

3.2.6. Effect of incorporation of *Sesbania rostrata* on rice

Field experiments was performed to study the effect of incorporation of both inoculated and uninoculated *Sesbania rostrata* along with control on rice growth, nitrogen fixation and establishment of *Azorhizobium*. The experiment was performed in Rice-B-Block fields, which was ploughed before sowing.

Sesbania rostrata seeds were inoculated with 48-hour old *Azorhizobial* cultures grown in YEM broth at room temperature. 4 cultures two root isolates (R_3 and R_4) and 2 stem isolates (S_1 and S_2) along with reference strain *A. caulinodans* ZB-SK-5 and control were mixed with seeds and charcoal powder. Seeds were rubbed with mixture with hands and shade dried. Seeds were sown in field in only 18 plots and other 18 plots were used as control plots. The super phosphate was applied along with seeds on the date of sowing. After 45 days sampling of *Sesbania rostrata* plants was done and following parameters were studied:

1. Plant Height: The plant height was measured in centimeters using a scale and measured from root-shoot junction to the meristem of each plant.
2. Plant biomass: Plants were carefully uprooted from field and biomass was taken of fresh plants on a weigh balance and expressed as kg per square meter per plot.
3. Root and shoot nodule number and mass: The stem and root nodules were carefully detached and fresh weight was taken. Nodules were dried at 80°C for 24 h and weighted on an electronic balance.

4. Nitrogenase activity: Root nitrogenase was carried out as described in section 3.2 5.2 (5) and was expressed as nM of C_2H_4 produced per plant per hour. While the shoot nitrogenase activity was performed using the Poropak R column. The region of shoot where maximum number of well-developed and healthy shoot nodules were present was cut (approximately 10 cm region) and put in vessel. The mouth of vessel was stoppered by rubber cork and 10 % (v/v) of air was injected out and same amount of acetylene gas was injected using a disposable syringe. The vessels were incubated for one hour at room temperature. Then 0.5/1.0 ml of sample gas was introduced in gas chromatograph and the number of units moved by indicator was counted. The calculated nitrogenase activity was measured and expressed as nM of C_2H_4 produced per 10 cm per hour.
5. Competitiveness of inoculated strains: The procedure was performed as described in section 3.2 5.1 (6)

After 45 days *Sesbania rostrata* plants were cut and incorporated in soil. The plots were flooded with water so as to help the decomposition of incorporated plants. Rice plants were transplanted onto these plots.

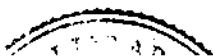
Rice plant nursery was prepared 15 days after sowing of *Sesbania rostrata* seeds. Rice seeds cv PR-115 were inoculated by *Azorhizobium* isolates (R₃, R₄, S₁, S₂ and ZB-SK-5) as per standard protocol of seed inoculation 6 nursery beds were prepared, 5 isolate inoculated and one control seed bed. One-month-old rice plants were then transplanted on plots. Before transplantation

seedlings treatment with respective cultures and following parameters were studied

1. Plant Height: Plant height was taken in cm using a scale
2. Tiller number: Number of tillers per plant was counted.
3. Panicle length: Panicle length was taken using a scale and expressed in cms.
4. Dry weight: Shoot and root dry weight was taken on a weight balance after drying at 60°C for 48 hrs.
5. Grain weight: Grain weight was measured on weight balance and expressed as gram per plant.
6. Para nodule number: Paranodules that appeared were counted with naked eyes and expressed as number per plant.
7. Nitrogenase activity: The procedure followed was as described in section 3.2 5.2 (5). The root nitrogenase activity was measured and expressed as nM of C₂ H₄ produced per plant per hour.
8. Yield: Rice yield was determined in Quintal per hectare and weighted on a weigh balance.
9. Competitiveness of inoculated strains: The rice roots were washed under tap water and surface sterilized by 0.1 % chloramine-T solution for 3 mins. 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 resistance 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.

The nitrogen was applied in split application manner. In *Sesbania* grown plots half of the recommended dose of nitrogen as urea was applied while full dose was applied in rest of plots.



Chapter-IV

RESULTS AND DISCUSSION

Rice (*Oryza sativa*) is one of the prominent staple diet of populations in developing world. For ever rising population in developing nations food resources are needed to be extended but short rises by use of agrochemicals plunders the soil of it's texture and properties and culminates to water and land pollution. Cereals particularly rice production is limited by supply of nitrogen and thus this crop needs high input of nitrogen fertilizers. Hence great deal of work is necessary for production of sustainable grain production techniques; one being Biological Nitrogen Fixation (Hardy, 1993). BNF benefits could be reaped in lowland rice soils where it could contribute 60Kg N ha⁻¹ and thus enhances N supply potential as fixed nitrogen become directly available to plant with little or no loss (Kannaiyan, 2001, Quraishi, 2000).

Green manuring is one of the several ways of adding nitrogen to soil fixed by legumes and offers most feasible, economical and environmentally safe way of nitrogen input for resource poor farmers (Kannaiyan, 1995; Ladha *et al*, 1992; Hiremath and Patel, 1998) *Azolla-Anabaena*, *Aeschynomene-Rhizobium* and *Sesbania rostrata* – *Azorhizobium* symbiosis could add considerable amount of nitrogen to support better rice production (Badaway *et al*, 1998; Rahman and Parson, 1999). Green manures increase soil macro as well as micro nutrients and bacteria associated to them increase their uptake by plant from the rhizosphere thus influencing seedling vigor and yield (Biswas *et al*, 2000; Biswas *et al*, 2000; Barraquio *et al*, 1997). Thus continuous green manure application improves soil

texture and properties that benefit subsequent cereals crops (Sharma *et al*, 1995). Rice rhizocoenosis involves diverse kind of bacteria associated with rice roots and water-logged basal stem portion that can fix nitrogen and a part of this is utilized by the rice plants (Wantanabe, 1986; Kannaiyan, 1998; Yanni, 1991; Roger and Ladha, 1992). Ladha *et al* (1989) has reported the natural endophytic association in rice roots by *A. caulinodans*. This rhizobium has been found to invade *S. rostrata* roots by crack entry at the point of emerging lateral roots (Dreyfus *et al*, 1988; N'Doye *et al*, 1994) and causes both root and stem nodulation. Thus it was reported that nod factors are required for efficient stem and root nodulation of *S. rostrata* that are arabinosylated and fucosylated (Lorquin *et al*, 1997; Mergaert *et al*, 1997; Prome 1996). Goormachtig *et al* (1999) reported accumulation of chalcone reductase homologous transcripts during development of stem borne nodules of *S. rostrata*. The leghaemoglobin increases in both root and stem nodules of *S. rostrata* (Kumar and Garg, 1998). Moreover it can fix nitrogen under free-living conditions even up to 3%v/v O₂ (Kitts and Ludwig, 1994). Hence it fulfills the suitability criteria for candidature of a possible future rice biofertilizer as it could encroach the rice cortex and could more or less intimately or directly supply the nitrogen fixed in nodule-like structures formed on the rice roots as a result of endophytic establishment (Websters *et al*, 1997). The hypertrophies or paranodules of cereals and non-legumes invaded by these bacteria or other diazotrophs could possibly increase the nitrogen uptake by the plant (Kennedy and Tchan, 1992; Khokar and Quraishi, 1997).

The present investigation was undertaken to observe the effect of phytohormones i.e. 2,4-D and NAA and role of signal compounds i.e. Naringenin and Acetosyringone along with inoculation of *A. caulinodans* isolates on various plant growth parameters and paranodule induction on rice roots. Histological studies of microtomic sections of rice roots revealed the endophytic invasion and colonization by *A. caulinodans* of rice roots. The isolates were screened by pot and field experiments on *S. rostrata* for their efficiency of nodulation and nitrogen fixation. Field experiment regarding effect of *A. caulinodans* under natural conditions on rice growth parameters and yield was also performed. The results of different experiments conducted under laboratory and field conditions are presented in this chapter along with discussions on critical observations.

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*

Nitrogen fixing nodules are a prominent feature of legume roots but stem nodulation was observed by Dreyfus and Dommergues (1981) in *Sesbania rostrata*, a tropical legume. A total of six isolates were obtained of which four were the root isolates viz. R₁, R₂, R₃ and R₄ while two were isolated from the stem nodules viz. S₁ and S₂.

4.1.2. Purification of the standard *A. caulinodans* isolates

The *A. caulinodans* isolates viz. *A. caulinodans* ZB-SK-5, *A. caulinodans* BB-SK-5 and *A. caulinodans* RB-SK-5 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 of the reference strains were studied (Table 1). The morphological studies revealed that the *A. caulinodans* cells are gram negative, motile rod cells having peritrichious flagella on solid medium. Among the *A. caulinodans* isolates both fast and slow growers were observed. *Rhizobium* isolates of *Sesbania rostrata* nodulate only root and were fast growers but *A. caulinodans* stem nodulating isolates of *S. rostrata* have both slow and fast grower types (Dreyfus *et al*, 1988) and nodulate both root and stem. Among root isolates R₁ and R₄ were found to be slow growers while all stem isolates were found to be fast grower. All root isolates except R₁ were acid producers while S₂ produced acid on BTB supplemented YEMA. Standard cultures were alkali producers and fast growers. The growth obtained on lactose agar remained white after flooding with the Benedict's reagent confirming isolates to be *A. caulinodans*.

4.1.4. Nitrogenase activity under free-living conditions

The in vitro nitrogenase activity of *A. caulinodans* isolates is presented in Table 2. The range of nitrogenase activity of isolates was recorded to be from 132.8-4331.7 nm C₂H₂ mg dry weight⁻¹h⁻¹. Among all isolates S₁ gave maximum activity of 4331.7 nm followed by S₂ 2623.6 nm C₂H₂ mg dry weight⁻¹h⁻¹. In root isolates R₃ showed 844.7 nm acetylene reduction activity followed by R₄ (202.7nM). The reference strain *A. caulinodans* ZB-SK-5 gave 776.25 nm acetylene activity. *A. caulinodans* is an obligate aerobe and could fix nitrogen under microaerophilic conditions (Dreyfus *et al*, 1988). Gerbhardt *et al* (1984)

Table 1: Morphological and Biochemical Characteristics of Azorhizobium isolates

Azorhizobium isolates	Gram's staining	Growth Pattern on YEMA	Growth on Congored supplemented YEMA	Colony Colour on BTB supplemented YEMA	Growth on Hofer's Alkaline Broth	Colony colour on lactose agar after flooding with Benedict's reagent
R1	(-ve)	Slow	SW	Alkaline	NG	White
R2	(-ve)	Fast	SLR	Acidic	VLG	White
R3	(-ve)	Fast	SLR	Acidic	VLG	White
R4	(-ve)	Slow	SDR*	Acidic	VLG	White
S1	(-ve)	Fast	SDR*	Alkaline	NG	White
S2	(-ve)	Fast	SDR*	Acidic	NG	White
ZB-SK-5	(-ve)	Fast	SDR*	Alkaline	NG	White
BB-SK-5	(-ve)	Fast	SDR*	Alkaline	NG	White

Slow -Growth after 2 days.

Fast - Growth after 24 hour.

SW - Slimy white.

* Growth seen after 3 days

SLR - slimy light red.

SDR - slimy dark red.

NG - No growth.

VLG- Very little growth.

Plate 2: Single colonies of *Azorhizobium caulinodans* isolate on YEMA medium

Plate 3: Growth of *A. caulinodans* isolate on YEMA supplemented with Bromothymol blue dye

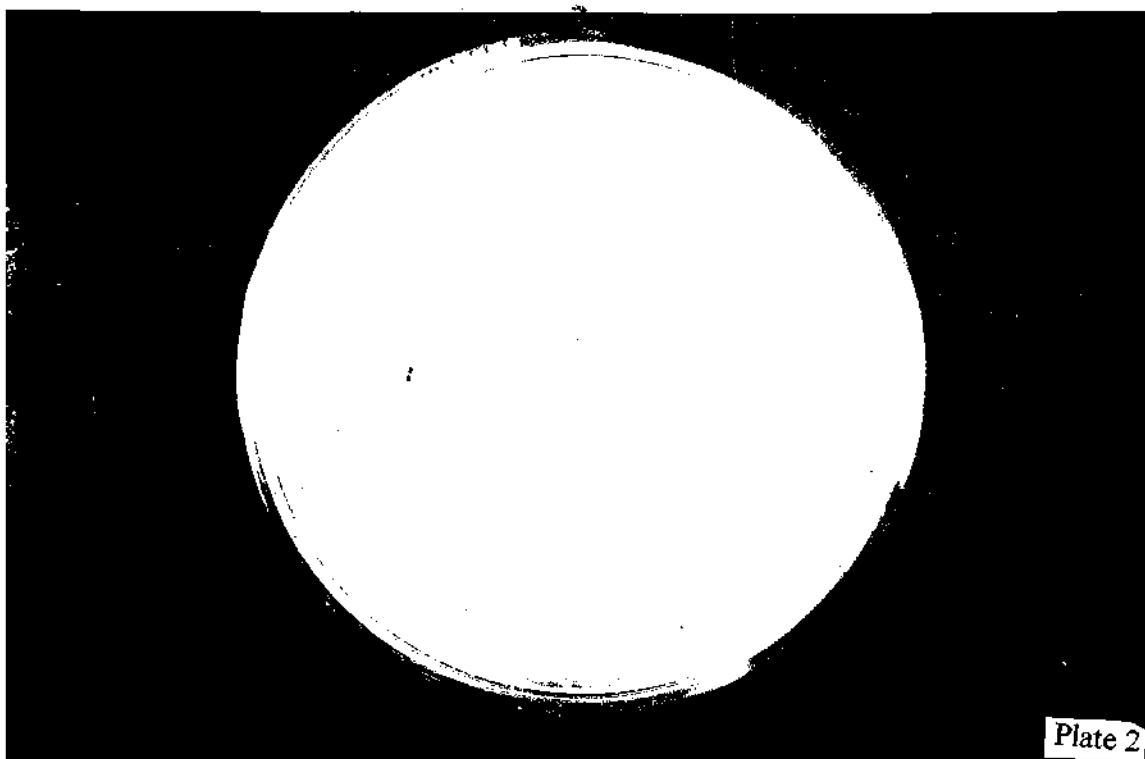


Plate 2

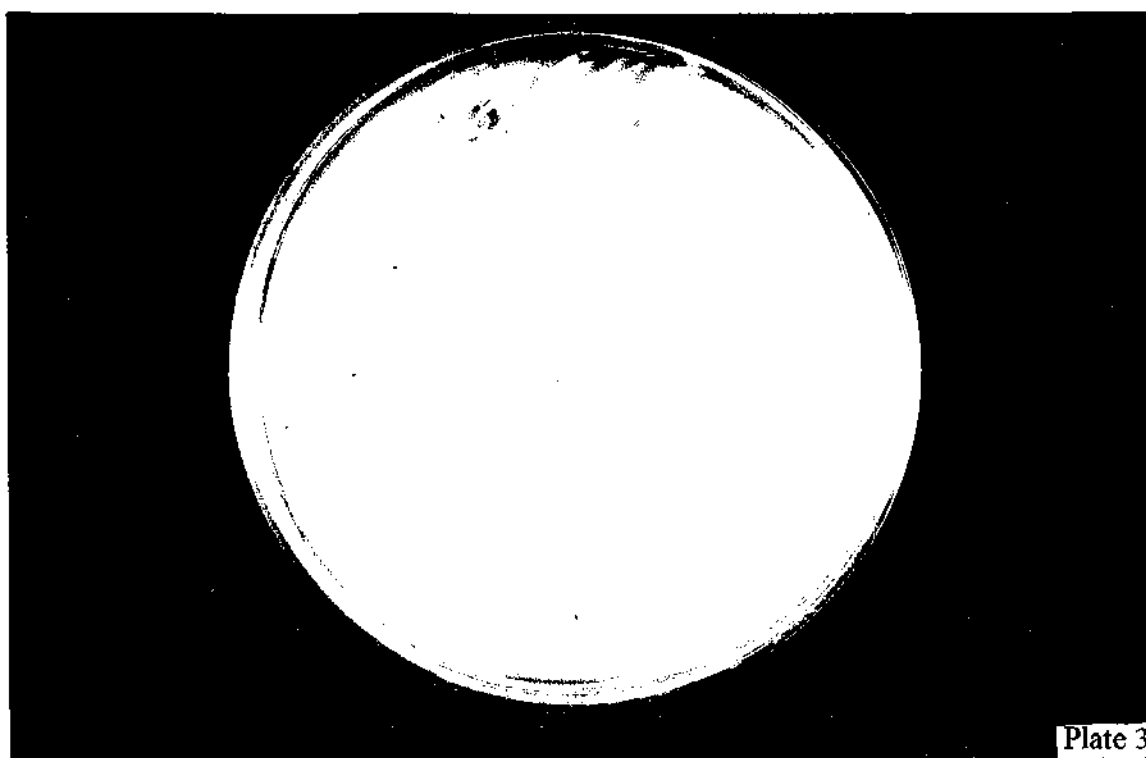


Plate 3

Table 2: Nitrogenase activity of various *Azorhizobium* isolates under free-living conditions

S.No	Isolates	Nitrogenase activity (nM C ₂ H ₂ /h/mg protein)
1	R1	171.00
2	R2	132.80
3	R3	844.70
4	R4	202.00
5	S1	4337.10
6	S2	2623.60
7	Reference	776.25

reported nitrogenase activity up to 2000 nm C₂H₂ mg dry wt⁻¹h⁻¹ under <9 µm dissolved oxygen continuous culture conditions. The root isolates thus recorded low nitrogenase activity compared to stem isolates. The difference in nitrogen fixing capability could be due to the presence of intrinsic genetic variabilities and acquired ecological factors.

4.1.5. Antibiotic resistance spectra of isolates

The intrinsic antibiotic resistance spectra of isolates to different antibiotics viz. ampicillin, chloramphenicol, kanamycin, neomycin, nalidixic acid and tetracycline were worked on (Table3). Among the root isolates all showed an identical resistance pattern except R₄ that showed resistance to ampicillin at all three working concentrations. R₄ was found to be resistant to neomycin and nalidixic acid at 10 and 20 µg/ml concentrations. The standard culture *A.caulinodans* ZB-SK-5 showed susceptibility for chloramphenicol and nalidixic acid at 100 µg/ml concentration. The stem isolate S₁ showed resistance to all antibiotics used except neomycin and nalidixic acid at 100 µg/ml concentration while S₂ showed resistance to all concentrations of nalidixic acid only. The intrinsic antibiotic resistance spectra could be used for strain identification and for ecological studies. Gupta *et al* (1983) studied the intrinsic antibiotic resistance spectra of *Rhizobium* isolates. The few similarities in antibiotic resistance spectra could be due to the fact that *A.caulinodans* isolates that were isolated from the field harbored almost same resistance plasmids.

Table 3: Antibiotic resistance spectra of *Azorhizobium* isolates

Azorhizobium isolates	Antibiotics (ug/ml)																															
	Ampicillin						Chloramphenicol						Neomycin						Tetracycline				Kanamycin				Nalidixic acid					
	10		20		50		100		10		20		50		100		10		20		50		100		10		20		50		100	
R1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
R2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
R3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
R4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
S1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
S2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
ZB-SK-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

+ Growth
 - No Growth
 Slight Growth

4.2 Studies on inoculation of *A.caulinodans* isolates and the effect of growth regulators and signal compounds on rice.

Four isolates two each of root and stem, of *A.caulinodans* were used for further studying the effect on root/shoot growth, total biomass, nodule like structure appearance and nitrogenase activity in rice seedlings cv PR-115.

4.2.1 Effect of growth regulators and *A.caulinodans* isolates inoculation

Growth regulators or phytohormones such as auxins and cytokinins possess a prominent role in development of root nodules in legume plants as they stimulate cortical cell division (Bauer, 1981; Bauer *et al*, 1985; Thinmann, 1936). Nodule formation initially involves the root hair curling mechanism that occurs due to impropportionate cell division triggered due to excretion of auxins/cytokinins by the invading rhizobia at the preinfection foci (Libbenga *et al*, 1973). Experimental inhibition of auxin transport by using 2,3,5-triidobenzoic acid causes pseudonodule induction on soybean roots (Hirsch *et al*, 1989). The role of growth regulators in basic initial interaction for the initiation of cell division at preinfection foci in legumes suggested the pathway for their use in induction of nodule like structures in non-legumes. Kennedy and Tchan (1992) and Francisco and Akao (1993) observed paranodule induction by auxin application and rhizobial inoculation in cereals.

Direct inoculation of several rhizobia could also result in nodulation in non-legumes particularly cereals (Ladha and Huang, 1997). Paranodule induction was achieved by Al-Mallah *et al* (1989a) by treating rice seedling roots with hydrolytic enzyme treatment that partially hydrolyzed the cell wall material thus

facilitating entry of rhizobia intercellularly. However, the rate of nodule like structure induction was more with increased stable paranodes inhabited by rhizobia. Pseudonodule or modified lateral root induction could be achieved by NAA/IAA application in cereals as wheat (Kennedy, 1992). However, application of 2,4-D or NAA at various concentrations along with *A.caulinodans* inoculation resulted in increased biomass of rice seedlings (Amutha and Kannaiyan, 1999). Treatment of auxins along with hydrolytic enzyme mixture and *A.caulinodans* increases short thickened lateral roots and paranodes due to invasion of *A.caulinodans* through fissures created during lateral rootlet emergence (Senthil Kumar, 2000). Kannaiyan (1998) reported *Azorhizobium caulinodans* invasion in rice roots by crack entry and later paranodule colonization in xylem.

4.2.1(a) Effect of 2,4-D on rice PR-115

4.2.1.1 Root growth.

Azorhizobium caulinodans isolates inoculation resulted in decreased root length over the uninoculated control (Table 4 and fig.1). 2,4-D at 3.0 ppm concentration showed maximum decrease in root length in all treatments in comparison to uninoculated control (Plate 4 and 5). S₁ among stem isolates showed maximum decrease in root length while R₄ gave short root among the root isolates. Thus inoculation of azorhizobial isolates resulted in decrease in root length ranging from 15.0-10.0 cm. Addition of 2,4-D at all concentrations resulted in stunted root growth. Moreover inoculation of azorhizobial isolates along with 2,4-D at all concentrations resulted in shortening of root in rice PR-115. This root

Table 4: Effect of 2,4-D and inoculation of *A.caulinodans* isolates on root growth of rice seedlings cv PR-115

Treatments	Root Length (cm)			
	2,4-D (ppm)			
	0.0	0.5	1.5	3.0
Control	16.75	14.50	11.20	10.70
R3	15.00	11.20	9.54	7.63
R4	11.50	8.94	7.93	5.50
S1	10.00	6.25	5.00	3.24
S2	13.50	6.03	5.45	4.90
ZB-SK-5	15.00	10.67	9.37	7.59

Figure 1

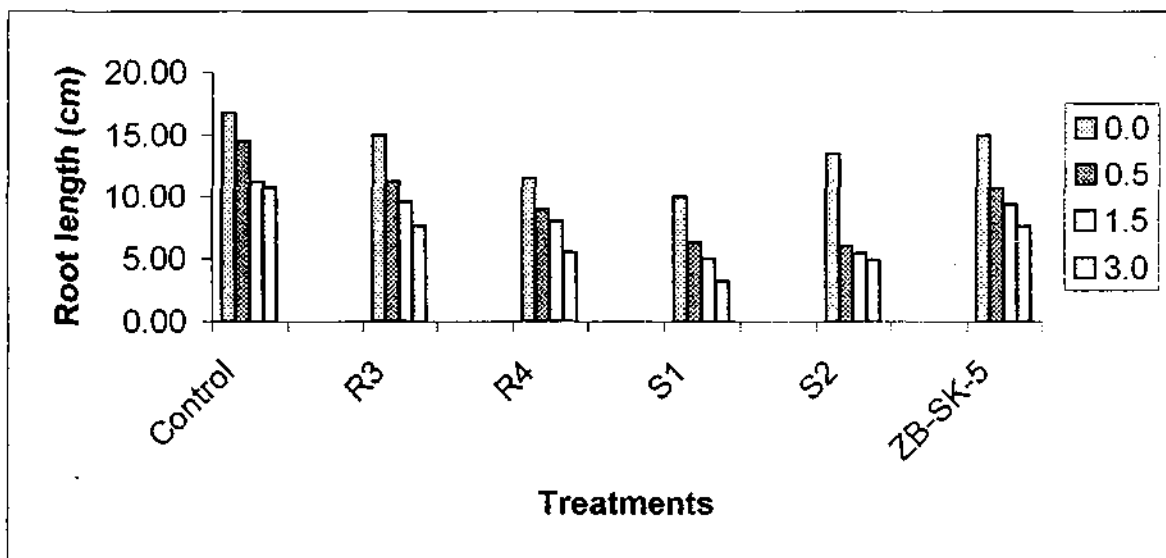


Plate 4: Effect of addition of 2,4-D at different concentrations on rice cv PR-115

Plate 5: Effect of inoculation of *A. caulinodans* isolate S₁ in presence of 2,4-D (0.5 ppm) on rice cv PR-115



Plate 4

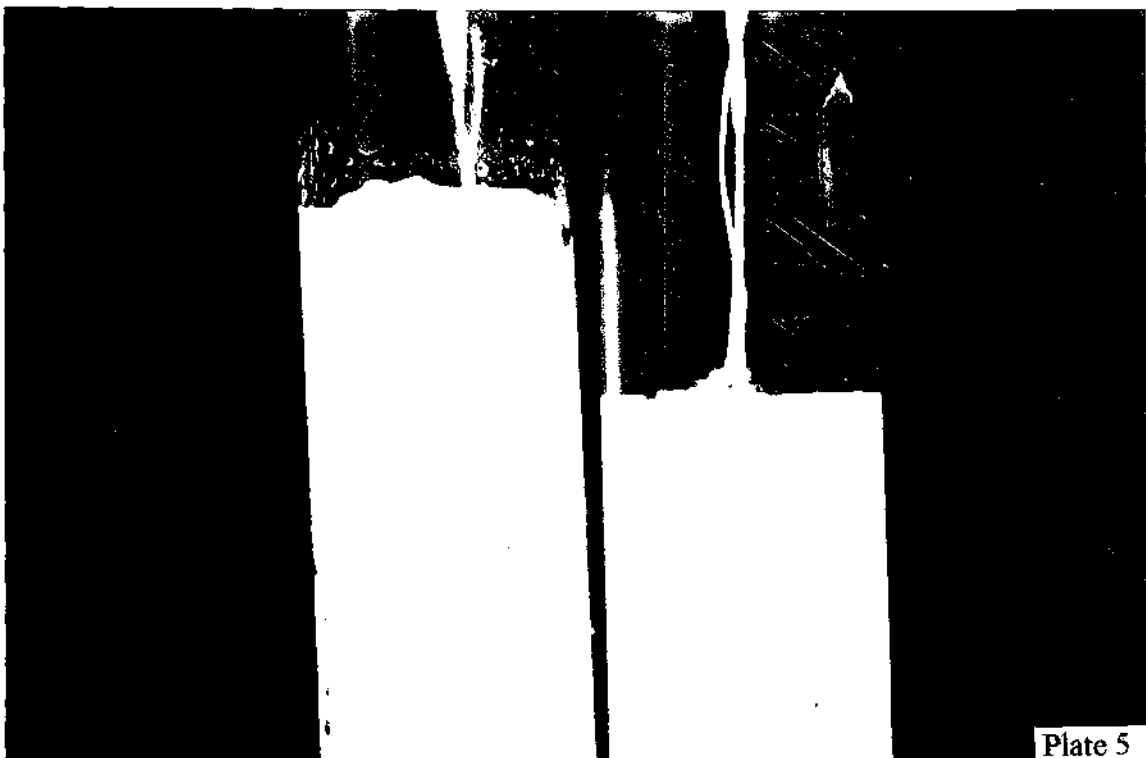


Plate 5

shortening occurs due to increased auxins in vicinity of roots that enhances the lateral rootlets to appear while shortens the roots.

4.2.1.2 Shoot growth

Inoculation of all azorhizobial isolates along with the standard culture resulted in increase in shoot length in rice PR-115 that ranged from 16.5-18.9 cm (Table 5 and fig. 2). 2,4-D at all concentrations except at 0.5 ppm concentration produced a decrease in shoot length. S₁ isolate inoculation at 1.5 ppm concentration of 2,4-D gave maximum shoot height followed by S₂. Among root isolates inoculation of R₄ isolate alone resulted in maximum shoot length (18.0cm) but at 1.5 ppm concentration shoot length decreased. Thus *Azorhizobium caulinodans* isolates inoculation resulted in subduing the effect of 2,4-D addition at all concentrations which was in accordance to that reported by Amutha and Kannaiyan (1999).

4.2.1.3 Nodule like structure formation

The results are presented in Table 6 and figure 3. S₁ isolate produced maximum number of paranodules at 1.5 ppm concentration among all isolates and the reference strain used for inoculation. Inoculation of S₁ isolate alone resulted in formation of paranodules (Plate 6 and 7). R₄ recorded maximum number of nodule like structures in root isolates at 3.0ppm concentration. The results are supported by work done by Kennedy and Tchan (1992). Francisco and Akao (1993) reported nodule like structures in 2,4-D alone treatments were irregularly lobed while the 2,4-D and *Rhizobium* treatment resulted in more globose paranodules that show rhizobial colonization (Nie *et al*, 1992). IAA

Table 5: Effect of 2,4-D and inoculation of *A.caulinodans* isolates on shoot growth of rice seedlings cv PR-115

TREATMENTS	Shoot Length (cm)			
	2,4-D (ppm)			
	0.0	0.5	1.5	3.0
Control	15.0	15.5	14.3	12.5
R3	17.5	15.0	17.0	15.5
R4	18.0	17.5	17.6	16.0
S1	18.9	17.7	18.9	17.2
S2	18.5	17.6	18.0	17.6
ZB-SK-5	16.5	17.0	17.1	16.2

Figure 2

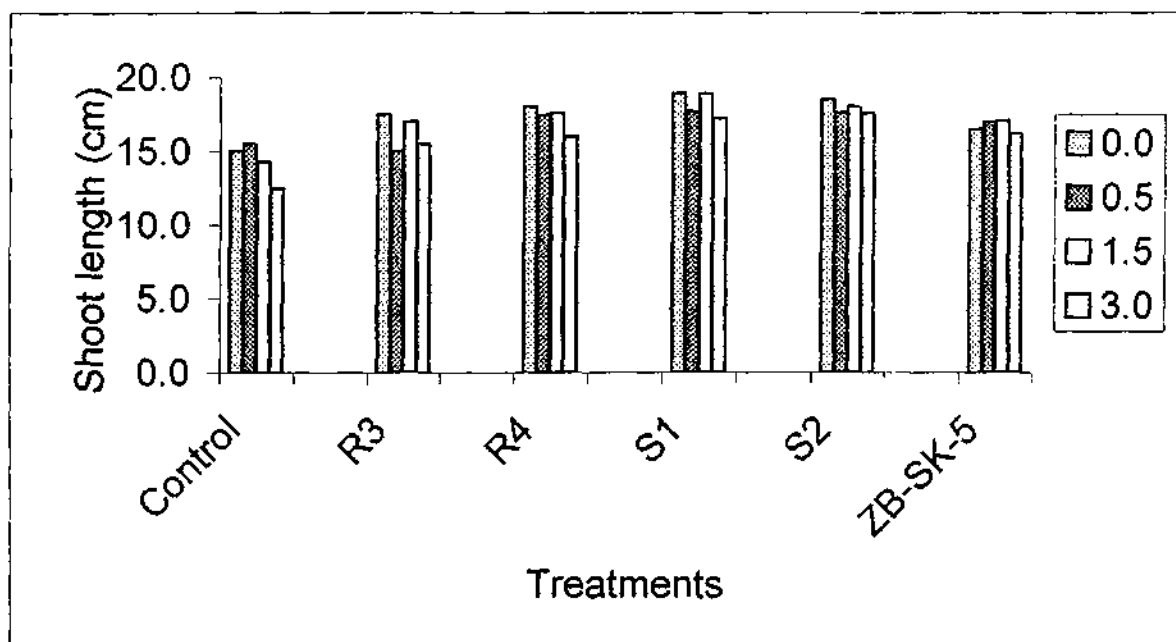


Table 6: Effect of 2,4-D and inoculation of *A. caulinodans* isolates on the nodule-like structure formation on rice seedlings cv PR-115

Treatments	Number of paranodes formed per plant			
	2,4-D(ppm)			
	0.0	0.5	1.5	3.0
Control	-	-	-	-
R3	-	3.0	6.5	4.4
R4	1.5	8.4	14.7	15.6
S1	4.0	15.9	19.0	18.3
S2	3.2	13.8	16.6	16.8
ZB-SK-5	1.0	13.0	14.4	15.0

Figure 3

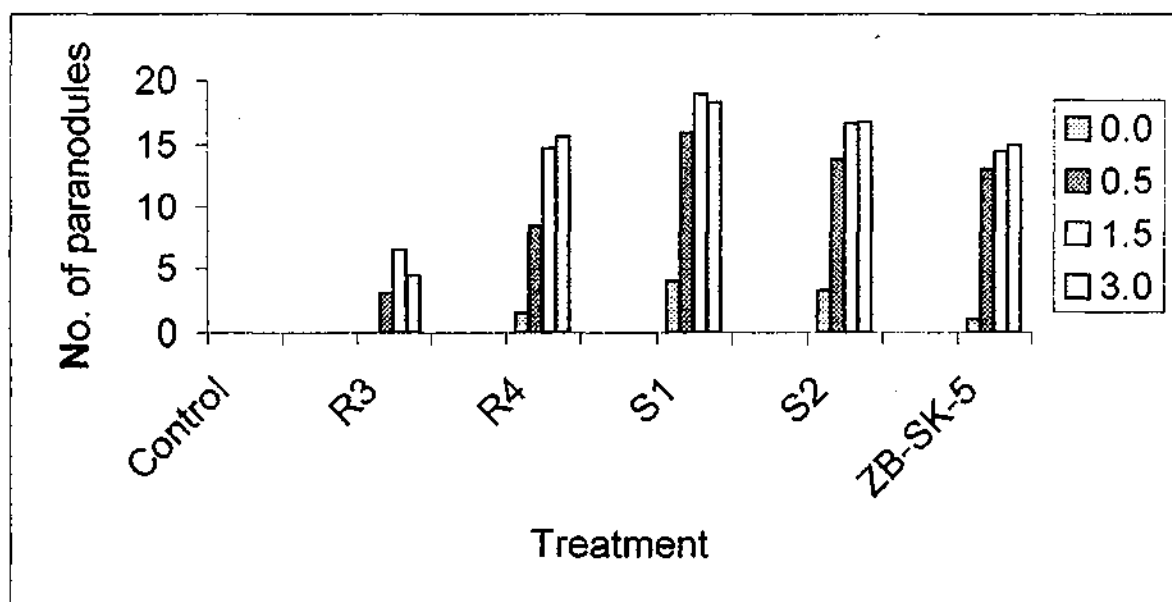
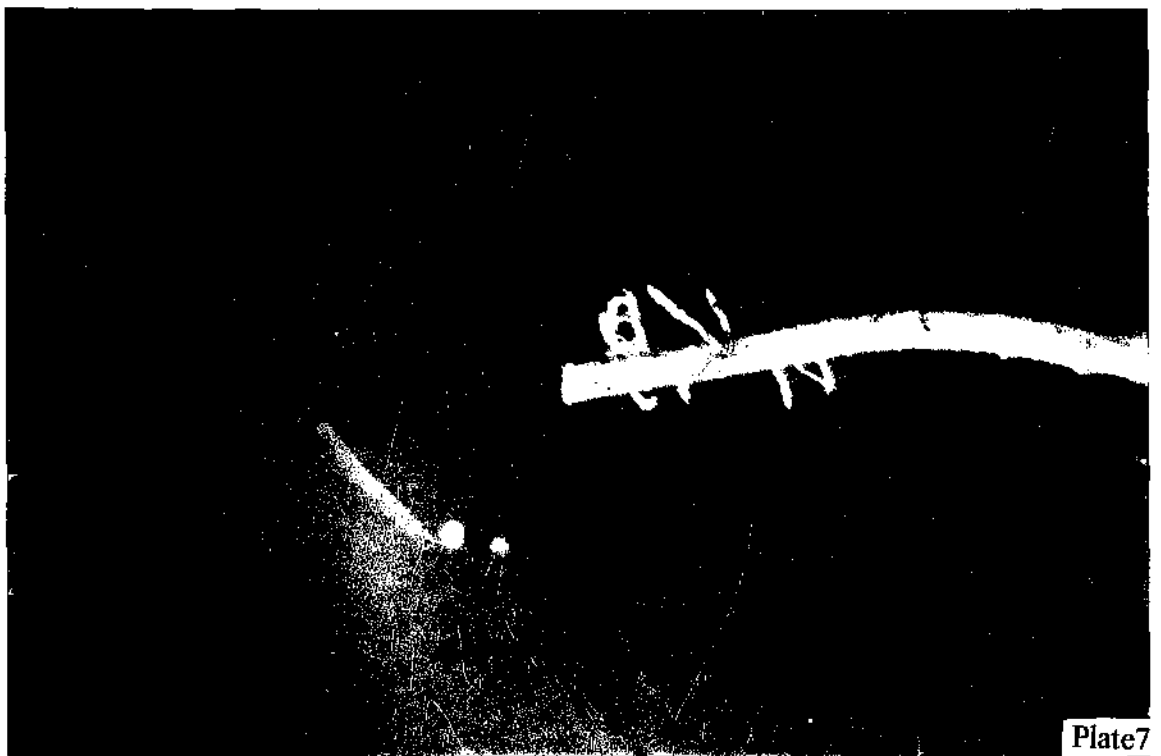


Plate 6: Close up view of the changed morphology of the rice roots treated with 2,4-D at 01.5 ppm concentration

Plate 7: Close up view of paranodule structures formed on rice roots after treatment with 2,4-D at 1.5 ppm and inoculation with S₁ isolate



treatment in wheat roots also resulted in paranodule formation (Chen *et al*, 1992). Amutha and Kannaiyan (1999) reported increase in number of paranodules in rice var ADT-36 and TKM-9.

4.2.1.4 Lateral rootlet number

The results are presented in Table 7 and figure 4. Maximum number of lateral rootlets were recorded in *A.caulinodans* ZB-SK-5 in inoculated seedlings followed by S₁ in uninoculated control plants the number of lateral rootlet was minimum. Addition of 2,4-D at all concentration caused formation of short thickened lateral rootlets. The azorhizobial isolate inoculation resulted in compensating the bad effect of 2,4-D addition to a certain extent. Similarly, Rolfe and McIver (1994) reported induction of modified root outgrowths by 2,4-D application in wheat/rice plants due to induction of meristems.

4.2.1.5 Fresh weight and Dry weight.

An increase was observed in azorhizobial isolate inoculated rice seedlings over the uninoculated control (Tables 8 and 9 and figures 5 and 6). S₁ stem isolate produced maximum fresh weight and dry weight followed by S₂ among isolate alone inoculations. A concentration of 1.5ppm of 2,4-D showed maximum fresh weight and dry weight in treated seedlings. Again S₁ isolates excelled by producing maximum fresh weight and dry weight followed by S₂ at 1.5 ppm concentration. Similar reports are being given by Chandrasekar and Kannaiyan (1995) showed that the inoculation of *A.caulinodans* along with growth regulator 2,4-D increased total biomass of rice, maize and sorghum seedlings.

Table 7: Effect of 2,4-D and inoculation of *A.caulinodans* isolates on lateral root formation in rice seedlingscv PR-115

Treatment	No.of lateral rootlet formed per plant			
	2,4-D(ppm)			
	0.0	0.5	1.5	3.0
Control	80.0	65.0	57.3	45.5
R3	95.5	67.0	53.5	40.0
R4	97.0	63.5	59.6	39.5
S1	100.1	65.0	50.3	39.6
S2	99.5	63.0	59.5	41.0
ZB-SK-5	105.0	60.0	47.5	44.6

Figure 4

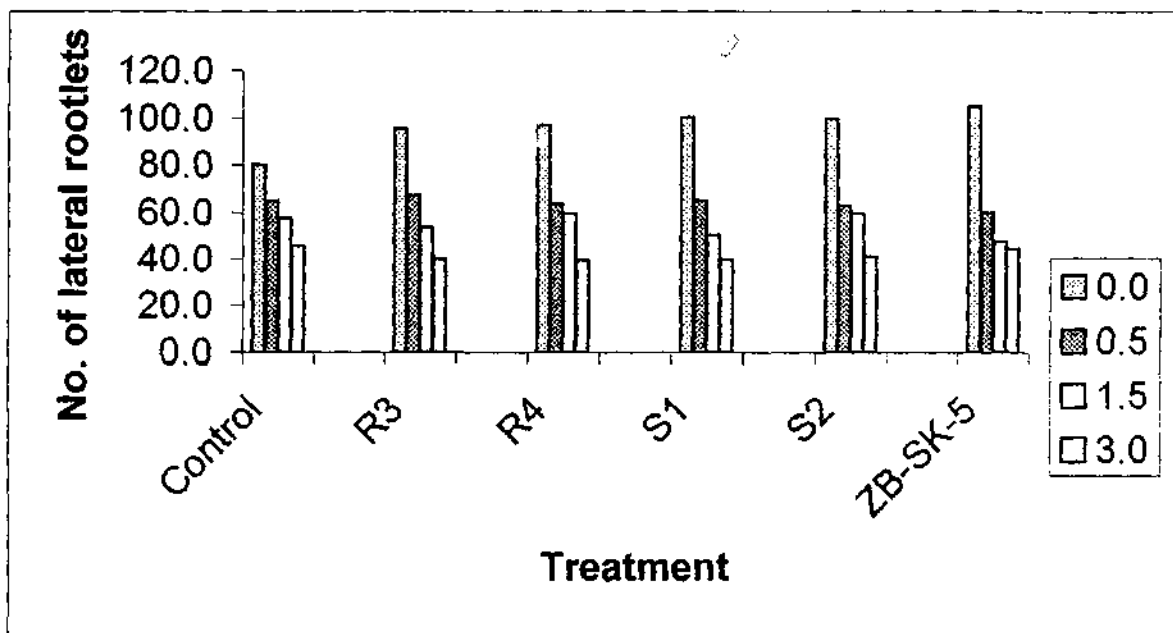


Table 8: Effect of 2,4-D and inoculation of *A.caulinodans* isolates on fresh weight of rice seedlings cv PR-115

Treatments	Fresh Weight (g/plant)			
	2,4-D(ppm)			
	0.0	0.5	1.5	3.0
Control	0.047	0.052	0.048	0.045
R3	0.061	0.054	0.063	0.061
R4	0.063	0.060	0.067	0.063
S1	0.065	0.060	0.072	0.068
S2	0.063	0.058	0.068	0.065
ZB-SK-5	0.062	0.054	0.065	0.061

Figure 5

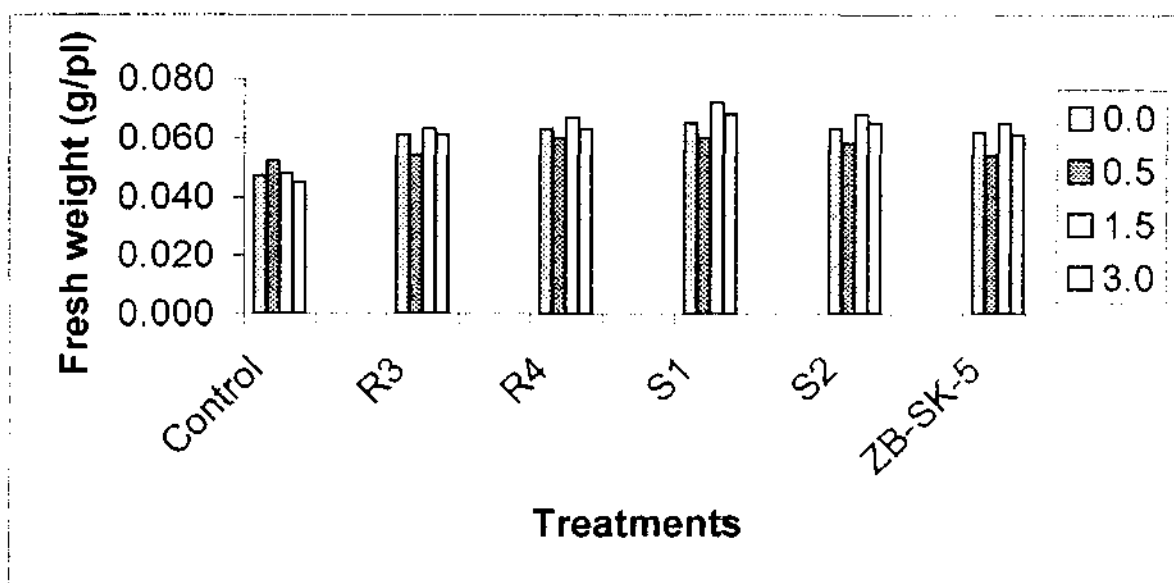
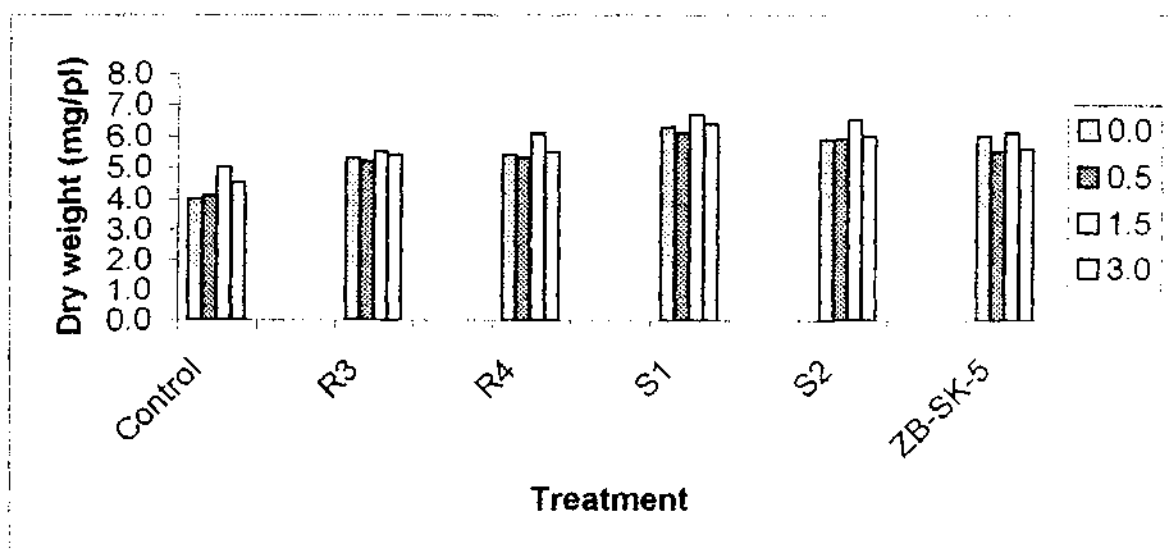


Table 9: Effect of 2,4-D and inoculation of *A.caulinodans* isolates on dry weight of rice seedlings cv PR-115

Treatments	Dry Weight (mg/pl)			
	2,4-D (ppm)			
	0.0	0.5	1.5	3.0
Control	4.0	4.1	5.0	4.5
R3	5.3	5.2	5.5	5.4
R4	5.4	5.3	6.1	5.5
S1	6.3	6.1	6.7	6.4
S2	5.9	5.9	6.5	6.0
ZB-SK-5	6.0	5.5	6.1	5.6

Figure 6



4.2.1.6 Nitrogenase activity

No nitrogenase activity was observed in uninoculated control plants (Table 10 and figure 7). The nitrogenase activity ranged from 2.8-14.1nM C₂H₂ plant⁻¹ 20h⁻¹ with S₁ showing the maximum activity. This parameter was observed to increase upon increasing the concentration of 2,4-D. Again S₁ treated seedlings gave maximum nitrogenase activity at all 3 concentrations of 2,4-D. Amutha and Kannaiyan (1999) have reported increased nodulation and nitrogenase activity by *A.caulinodans* inoculation and treatment with 2,4-D at 3.0 ppm concentration and Kinetin at 5.0 ppm in rice, cumbu, ragi and maize seedlings.

4.2.1.7 Histological studies

The histological analysis revealed the presence of azorhizobia on the surface and intercellularly in the rice cortex (Plate 8). The bacteria moved inside through rupture in the epidermis facilitating the crack entry of azorhizobia in the cortex of the rice roots which are in accordance to the work by Buvana (2001) who reported the intercellular crack entry of *A.caulinodans* ZB-SK-5 in the rice and maize roots in presence of 2,4-D + Hydrolytic enzymes mixture.

2,4-D at all concentrations resulted in decrease in root length and lateral rootlet number and increased the shoot length, number of paranodes, fresh and dry weight and the nitrogenase activity. S₁ excelled among all isolates in all parameters and 1.5ppm concentration produced better results over all concentrations regarding first three parameters.

Table 10: Effect of 2,4-D and inoculation of *A.caulinodans* isolates on nitrogenase activity of rice cv PR-115

Treatments	Nitrogenase activity (nM/plant/20 h)			
	2,4-D(ppm)			
	0.0	0.5	1.5	3.0
Control	-	-	-	-
R3	-	7.6	7.9	8.0
R4	-	12.1	12.7	12.8
S1	4.3	13.3	13.6	14.1
S2	4.0	12.5	13.0	13.6
ZB-SK-5	2.8	12.2	12.5	13.0

Figure 7

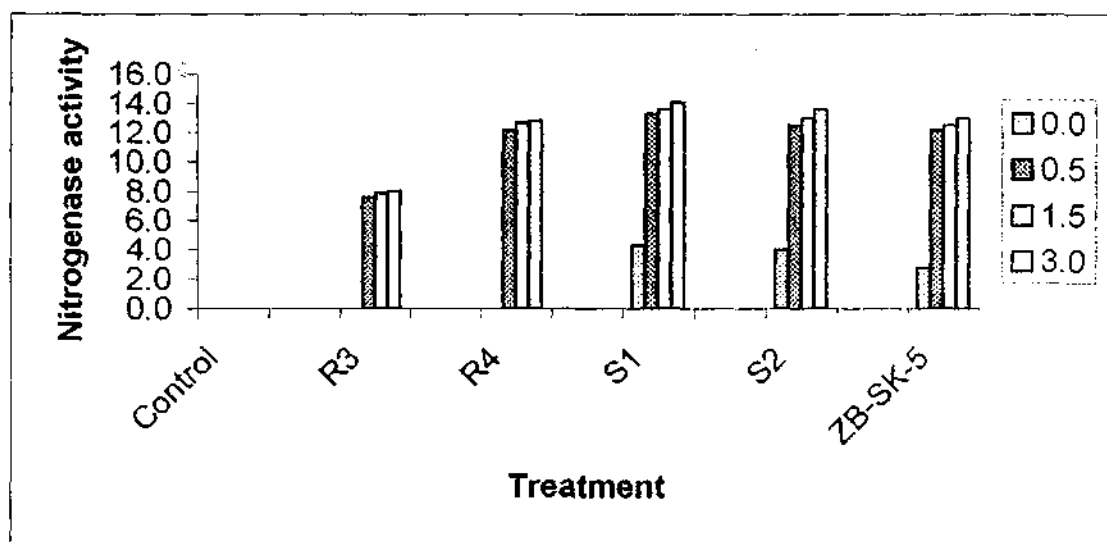
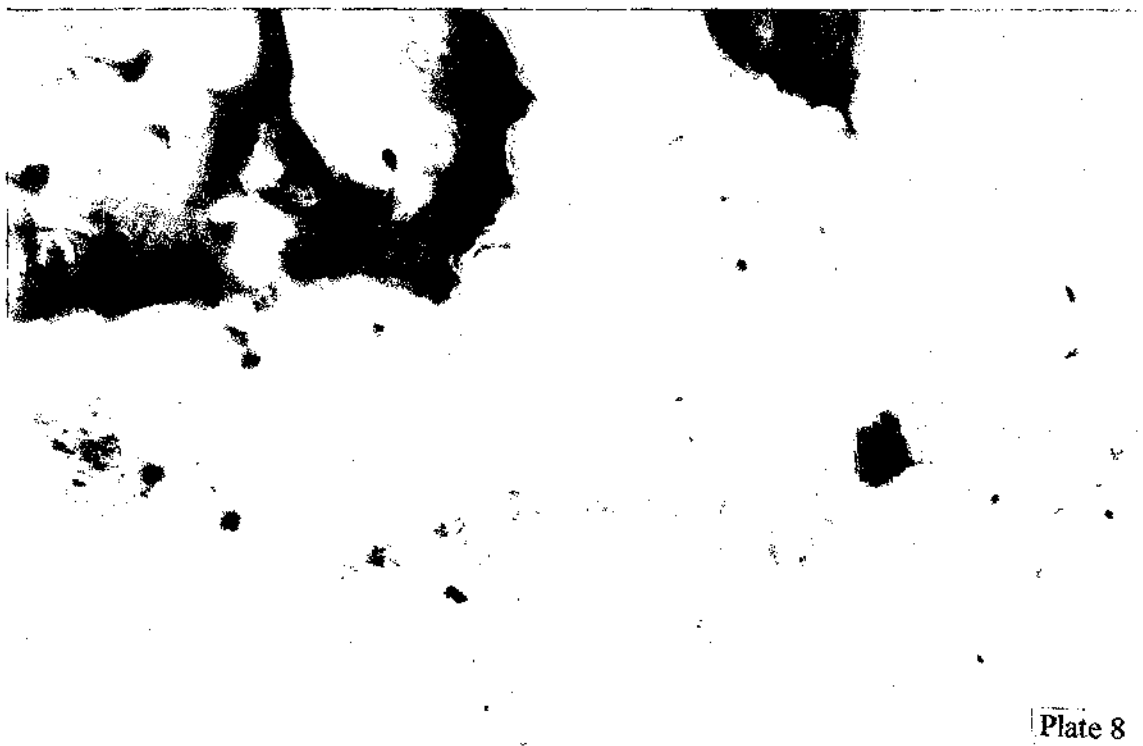


Plate 8: Stained microtomic section of modified lateral root of rice treated with 2,4-D and *A. caulinodans* isolate S₁



4.2.1(b) Effect of NAA on rice seedlings cv PR-115

4.2.1.8 Root growth.

The results are presented in Table 11 and figure 8 show increase in root length upon inoculation of azorhizobial isolates in comparison to uninoculated control (Plate 9). S₁ gave maximum root length followed by R₄ while the reference strain gave the maximum root length of 13.3 cm. As a general trend NAA treatment at all concentrations resulted in decrease in root length. The overall decrease in root length ranged from 6.3-9.2 cm among various isolates and all 3 concentrations of NAA. Maximum decrease in root length was observed in *A.caulinodans* ZB-SK-5 at 3.0 ppm and 5.0 ppm concentration of NAA while both R₃ and ZB-SK-5 gave decrease in root length of 6.3 cm at 7.0 ppm concentration of NAA. The decrease in the root length could be due to the increase in the amount of NAA present in the medium in which the plant was growing.

4.2.1.9 Shoot length

Addition of various azorhizobial isolates resulted in increase in shoot growth that ranged from 16.10-17.39cm, which was better than the uninoculated control (Table 12 and figure 9). NAA treatment at all concentrations resulted in decrease in shoot length in uninoculated controls while inoculation with *Azorhizobium* isolates compensated the decrease in shoot length. Maximum shoot height was recorded by S₁ isolate at both 3.0 and 5.0 ppm concentrations. NAA at 3.0 ppm concentration gave better results than at rest two concentrations that aggravated to more decrease in shoot length. The increase in the

Table 11: Effect of NAA and inoculation of *A. caulinodans* isolates on the root growth of rice cv PR-115

Treatment	Root Length (cm)			
	NAA(ppm)			
	0.0	3.0	5.0	7.0
Control	13.0	8.5	6.3	4.2
R3	14.0	8.9	8.0	6.3
R4	13.9	9.0	7.6	6.5
S1	14.3	9.2	7.5	6.8
S2	13.7	9.0	7.4	6.8
ZB-SK-5	13.3	8.8	7.0	6.3

Figure 8

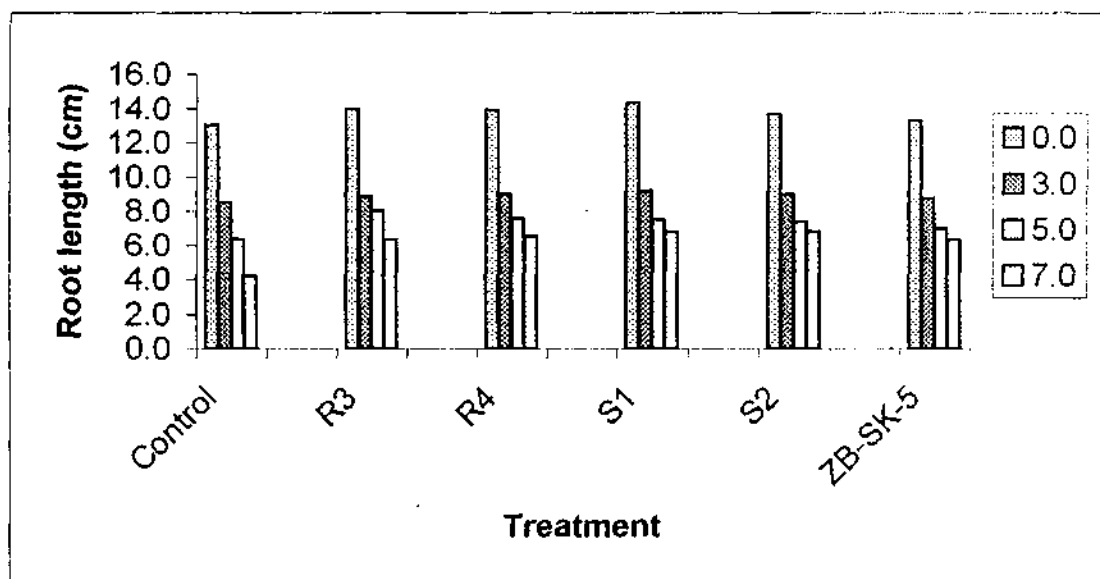
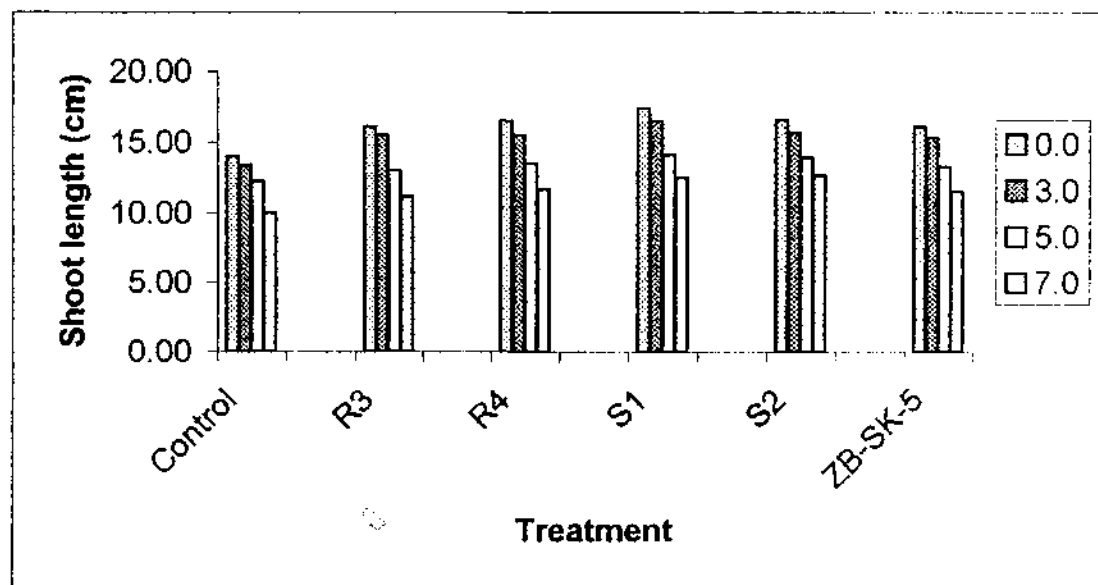


Table 12: Effect of NAA and inoculation of *A.caulinodans* isolates on the shoot growth of rice seedlings cv PR-115

Treatments	Shoot Length (cm)			
	NAA(ppm)			
	0.0	3.0	5.0	7.0
Control	14.00	13.36	12.20	10.00
R3	16.10	15.50	13.00	11.10
R4	16.50	15.50	13.50	11.65
S1	17.39	16.50	14.10	12.50
S2	16.62	15.70	13.91	12.65
ZB-SK-5	16.10	15.30	13.25	11.50

Figure 9



concentration of the NAA caused decrease in the shoot length due to the stunting effect of the phytohormone.

4.2.1.10 Paranodule formation

No paranodes were observed in the uninoculated control as well as isolate inoculated seedlings except S₁ inoculated rice roots that showed a single paranodule (Table 13 and figure 10). NAA treatment alone at all three concentrations produced no paranodule formation. Maximum number of paranodes were observed in S₁ isolate at all concentrations of NAA. A similar report has been given by Amutha and Kannaiyan (1999) who have reported paranodule induction due to NAA treatment and *A. caulinodans* inoculation in rice var. ADT-36 and TKM-9.

4.2.1.11 Number of lateral rootlet formed

Table 14 and figure 11 shows that inoculation of *A. caulinodans* isolates resulted in decrease in number of lateral rootlets per plant. Maximum lateral rootlets were formed in S₁ treated rice seedlings. Treatment with NAA at all concentrations resulted in decrease in number of lateral rootlet formation. Maximum decrease was observed in seedlings treated with 7.0 ppm concentration with all azorhizobial isolates. Similarly, Kennedy (1992) reported strongly modified lateral root formation in wheat seedlings upon NAA/IAA treatment.

4.2.1.12 Biomass

The results presented in Tables 15 and 16 and figures 12 and 13 show an increase in both fresh and dry weight in all NAA concentrations inoculated with

Table 13: Effect of NAA and inoculation of *A. caulinodans* isolates on the nodule-like structure formation on roots of riceseedlings cv PR-115

Treatments	No. of paranodes formed per plant			
	NAA(ppm)			
	0.0	3.0	5.0	7.0
Control	-	-	-	-
R3	-	2.0	10.5	15.0
R4	-	3.0	10.6	16.0
S1	1.0	18.0	22.5	27.5
S2	-	14.5	20.0	23.5
ZB-SK-5	-	13.0	19.5	23.0

Figure 10

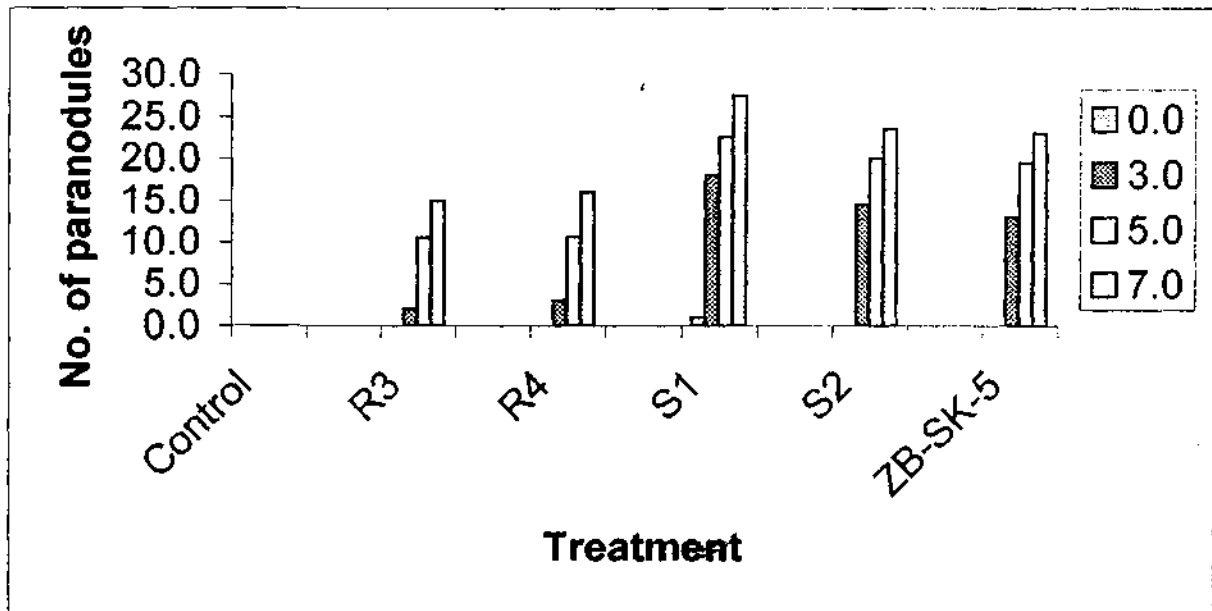


Table 14: Effect of NAA and inoculation of *A. caulinodans* isolates on number of lateral rootlet formed on rice seedlings cv PR-115

Treatments	No. of lateral rootlet per plant			
	NAA(ppm)			
	0.0	3.0	5.0	7.0
Control	78.5	81.6	67.5	35.4
R3	86.4	66.5	59.3	23.0
R4	89.3	69.3	58.2	24.0
S1	99.5	72.5	60.5	23.5
S2	90.3	70.2	59.0	22.2
ZB-SK-5	86.5	67.5	56.5	21.0

Figure 11

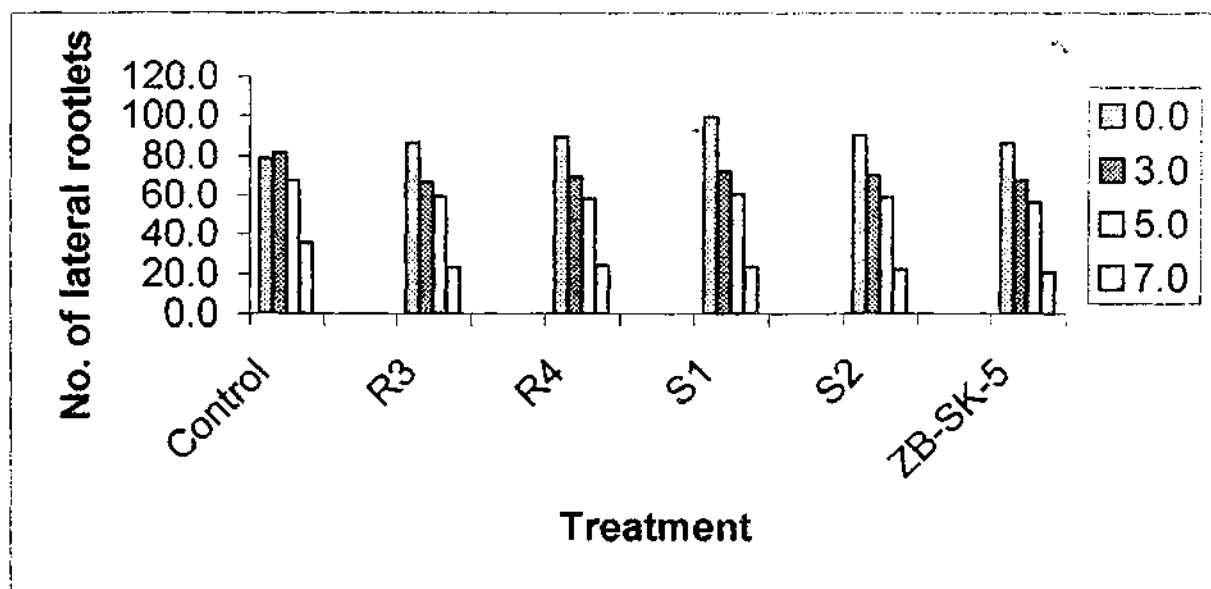


Table 15: Effect of NAA and inoculation of *A. caulinodans* isolates on fresh weight of rice seedlings cv PR-115

Treatments	Fresh Weight (g/plant)			
	NAA(ppm)			
	0.0	3.0	5.0	7.0
Control	0.044	0.040	0.036	0.030
R3	0.054	0.050	0.049	0.044
R4	0.058	0.053	0.050	0.047
S1	0.065	0.057	0.053	0.049
S2	0.063	0.056	0.053	0.047
ZB-SK-5	0.059	0.053	0.049	0.043

Figure 12

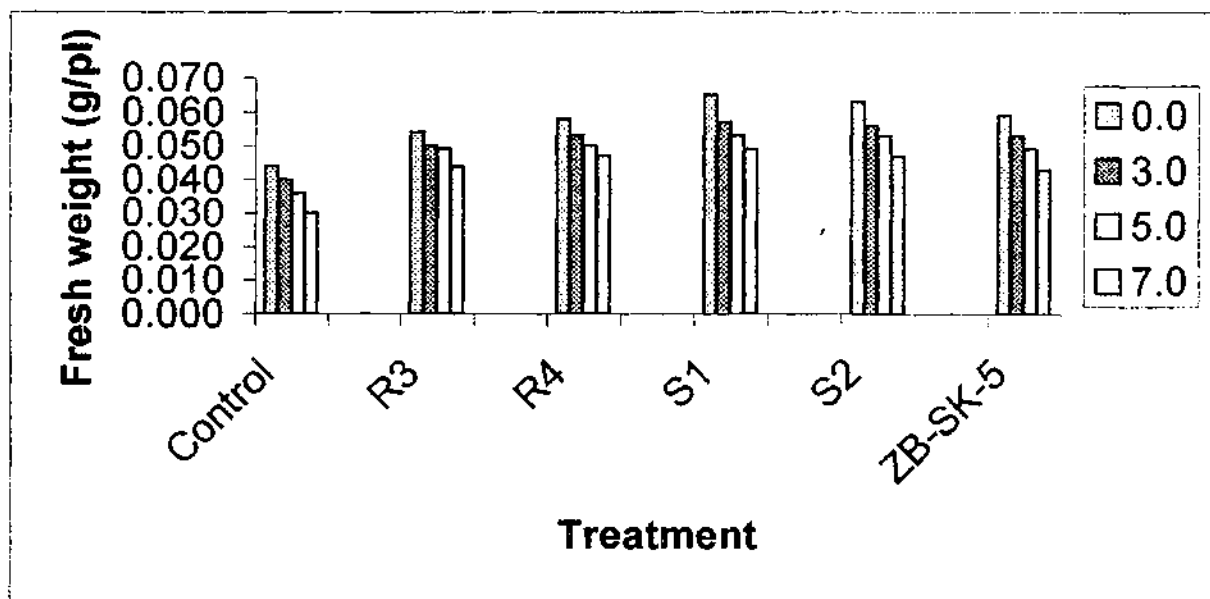


Table 16: Effect of NAA and inoculation of *A. caulinodans* isolates on dry weight of rice seedlings cv PR-115

Treatments	Dry Weight (mg/ plant)			
	NAA(ppm)			
	0.0	3.0	5.0	7.0
Control	4.8	4.2	3.8	3.2
R3	6.0	5.0	5.0	4.5
R4	6.5	5.5	4.9	4.5
S1	7.7	6.0	5.7	5.5
S2	7.1	6.0	5.5	5.0
ZB-SK-5	6.7	5.5	5.0	4.1

Figure 13

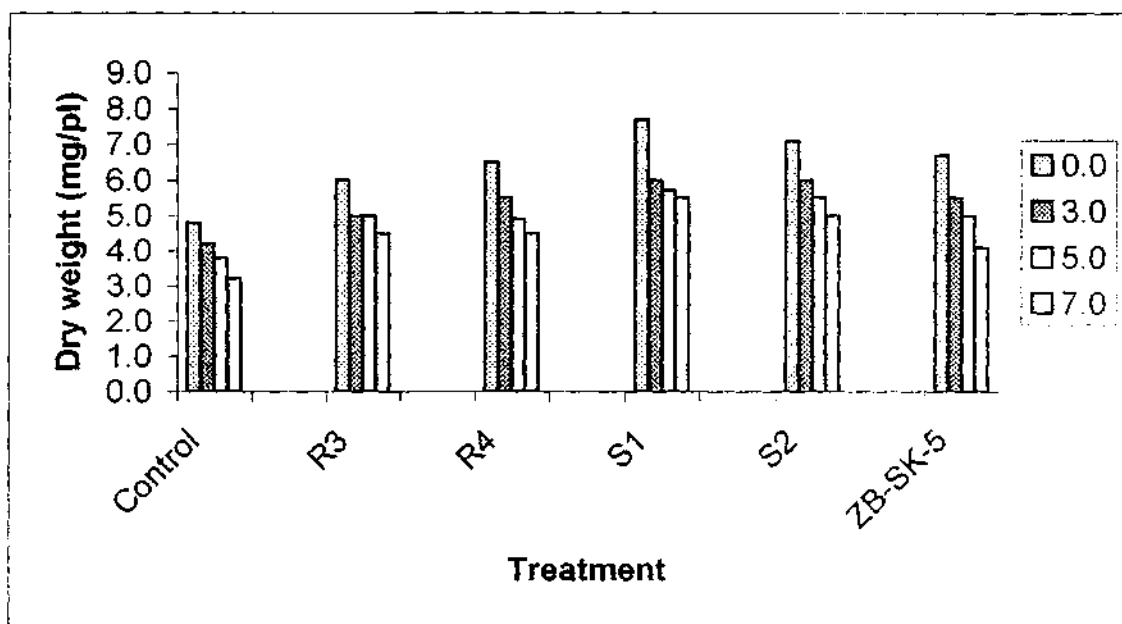


Plate 9: Effect of inoculation of *A. caulinodans* isolate in presence of NAA on rice cv PR-115

•

Plate 10: Stained microtomic section of rice root treated with NAA and *A. caulinodans* isolate



Plate 9



Plate 10

A.caulinodans isolates. Among the isolates inoculated seedlings maximum fresh and dry weight was recorded in S₁ followed by S₂. Among the three working concentrations of NAA 3.0 ppm gave better results over the other two concentrations. At 7.0 ppm maximum decrease in fresh and dry weight was observed. This clearly shows that increasing concentration above 3.0 ppm results decrease in these attributes but NAA treatment does increases fresh and dry weight with respect to uninoculated control plants. Thus Amutha and Kannaiyan (1999) have also reported the increase in the biomass of rice plants ADT-36 and TKM-9 due to treatment with NAA along with inoculation of *A.caulinodans* ZB-SK-5.

4.2.1.13 Nitrogenase activity

The nitrogenase activity is presented in Table 17 and figure14. It was observed to be maximum in seedlings treated with NAA at 7.0 ppm. In isolate alone seedlings no nitrogenase activity was recorded. Similarly no nitrogenase activity was recorded in the uninoculated seedlings in all 3 concentrations of NAA used. S₁ showed maximum at all 3 concentrations of NAA. The nitrogenase activity ranged from 6.7-16.6 nM C₂H₄ plant⁻¹h⁻¹.

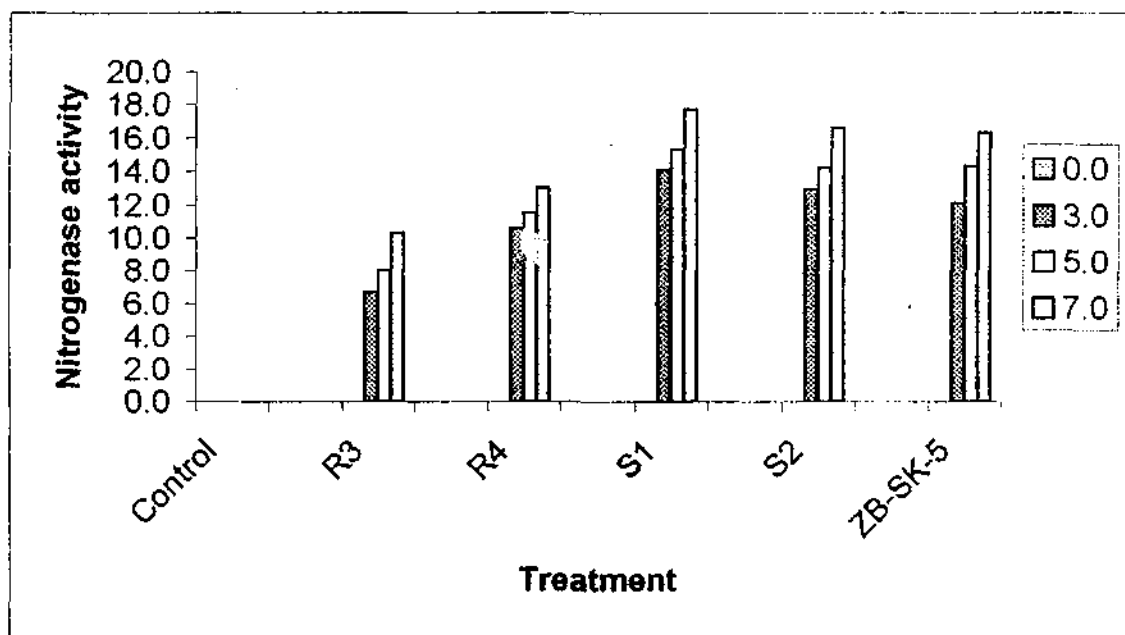
4.2.1.14 Histological studies

The histological studies of the stained microtomic sections revealed increased accumulation of *A.caulinodans* bacteria on the surface of the rice epidermis and intercellular entry in the cortical cells of the rice roots (Plate 10). Intercellular incorporation of the *A.caulinodans* ORS571 (pXLGD4) has been reported in rice roots treated with NAA + Naringenin + Hydrolytic enzymes by

Table 17: Effect of NAA and inoculation of *A. caulinodans* isolates on nitrogenase activity of rice seedlings cv PR-115

Treatments	Nitrogenase activity (nM/plant/20hr)			
	NAA(ppm)			
	0.0	3.0	5.0	7.0
Control	-	-	-	-
R3	-	6.7	8.0	10.3
R4	-	10.6	11.5	13.0
S1	-	14.1	15.3	17.7
S2	-	12.9	14.2	16.6
ZB-SK-5	-	12.1	14.3	16.3

Figure 14



Buvana (2001). The presence of phytohormone as NAA increases the number of lateral rootlets that thus increases the chances of the crack entry and encroachment from the ruptured epidermal margins around the emerging lateral rootlets.

NAA at all concentrations resulted in decrease in root and lateral rootlet number while an increase was recorded in shoot length, number of paranodules, fresh and dry weight and nitrogenase activity in all isolates. S₁ performed best among all and 3.0 ppm concentration gave better results.

4.2.2. Effect of signal compounds on rice seedlings PR-115

Signal compounds basically involve the secondary metabolic products or the phenolic compounds which are secreted in the rhizosphere of the plant. These phenolics involve various classes among which flavonoids included diverse kind of members that cause initial interaction events for nodulation (Denarie *et al*, 1992). Few flavonoids have been reported to be the endogenous polar auxin transport regulators (Jacobs and Rubery, 1988) as they lead to accumulation of auxins at a particular site and vice versa. Phenolics possess peculiar property of acting as chemo attractants (Peters and Verma, 1990) and work as transcriptional activators for several genes in various rhizobacteria and also act as carbon or energy source for rhizobia (Long, 1989,1996; Parker *et al*, 1985). The specificity related to phenolic excretion by roots of legume plants and their interaction with respective rhizobia leads to nodulation (Kapulnik *et al*, 1987). Nodulation could be extended to nonlegumes such as rice, wheat, sorghum, clover and maize by either using hydrolytic enzymes followed by PEG

treatment of roots (Al-Mallah *et al.*, 1989) that exposes the plasma membrane at tip of the root hairs enabling rhizobia to enter and invade. Some researchers have also reported formation of paranodules in nonlegumes if infected with *Aeschynomene* and *Parasponia Rhizobium*.

In present study Naringenin and Acetosyringone have been used as signal compounds to induce paranodules on rice PR-115. Even in legumes such as alfalfa symbiotic efficiency with *Rhizobium meliloti* can be enhanced by naringenin although it is non-inducer for *Rhizobium meliloti* nod genes. Naringenin is one of the most efficient inducer for nod genes expression *A.caulinodans* ORS571. The products of nod genes i.e. Nod factors are the chitooligosaccharides that are involved in intercellular crack entry infection in *Sesbania rostrata* (Goethals *et al.*, 1989). Naringenin and Daidzein at 5×10^{-5} concentration significantly stimulate LRC colonization by *Herbaspirillum seropediacae* up to very high levels while low concentrations of specific flavonoids significantly stimulated LRC colonization of *A.caulinodans* ORS571 in *Arabidopsis thaliana* (Gough *et al.*, 1997) *A.caulinodans* in presence of naringenin at 10^{-4} to 10^{-5} M concentration was found to increase number of seminal and adventitious roots in various rice varieties significantly if bacteria was inoculated 5 days after germination irrespective of amount of inoculum used (Gopalaswamy, 1998). Naringenin specifically elicit colonization of *A.caulinodans* in LRC's even at low concentration in wheat while no such reaction observed for *Rhizobium meliloti* (Websters *et al.*, 1997). O'Callaghan *et al.* (2000) reported effect of glucosinolates and flavonoids on root colonization of *Brassica napus* by

A.caulinodans ORS571. Rice roots do not produce flavonoid naringenin and thus introduction of a specific gene that produces naringenin would be beneficial for rice (Kannaiyan, 1998). Buvana (2001) reported the presence of naringenin at concentration of 1.93 ppm in maize root extract.

Acetosyringone act as a powerful signal molecule for several members of rhizobiaceae particularly *Agrobacterium tumifaciens*. Induction was found to depend upon the structure of the phenolic compound. Acetosyringone had the greatest activity and was found to be effective inducers (Bolton *et al*, 1986). Acetosyringone and α -hydroxy acetosyringone are released in the rhizosphere of the dicot plants and act as chemoattractants for *Agrobacterium tumifaciens* that degrades these compounds for deriving energy.

4.2.2(a) Effect of naringenin on rice seedlings PR-115

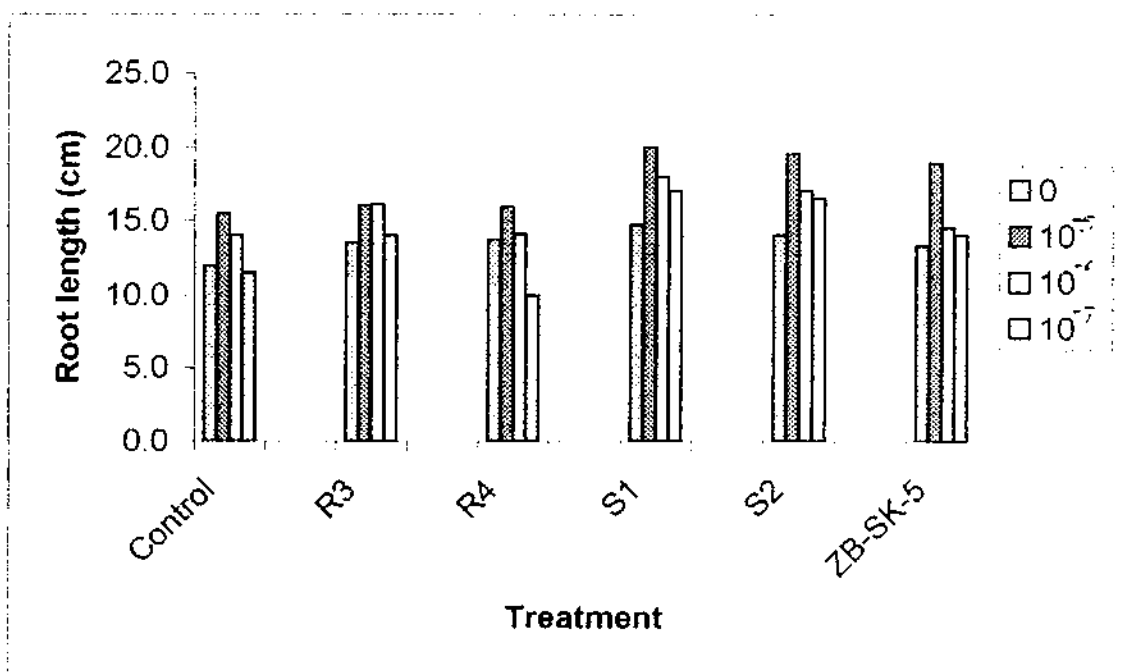
4.2.2.1 Root growth

The results are presented in Table 18 and figure 15 that clearly depicted enhanced root growth in naringenin + *A.caulinodans* isolate inoculated seedlings (Plate 11). Maximum root length was recorded at 10^{-5} M concentration among the three different concentrations of used in all the isolates inoculated. S_1 performed best at all naringenin concentrations among all other isolates. Inoculation of S_1 isolate alone resulted in increase in root length that was maximum among all other isolates inoculated. Buvana (2001) reported increase in root length on naringenin addition in the media which is in accordance to the above work.

Table 18: Effect of Naringenin and inoculation of *A. caulinodans* isolates on root growth of rice seedlings cv PR-115

Treatments	Root Length (cm)			
	Naringenin(M)			
	0	10^{-8}	10^{-6}	10^{-7}
Control	12.0	15.5	14.0	11.5
R3	13.5	16.0	16.1	14.0
R4	13.7	15.9	14.1	10.0
S1	14.7	20.0	18.0	17.0
S2	14.0	19.5	17.0	16.5
ZB-SK-5	13.3	18.9	14.5	14.0

Figure 15



4.2.2.2 Shoot growth

Table 19 and figure 16 shows an increase in length of rice shoot among the seedlings inoculated with *A.caulinodans*. 10^{-5} M concentration of naringenin produced maximum shoot height for all the isolates inoculated. S₁ inoculated seedlings gave best results in all treatments followed by S₂. The reference strain produced the minimum shoot length at all concentrations of naringenin used. Inoculation of *A.caulinodans* ORS571 pXLGD4 and naringenin on second and fifth day after germination increased root and shoot length significantly in *Sesbania rostrata* and cereals (Buvana, 2001).

4.2.2.3 Nodule like structure formed

The results of paranodule formation are presented in Table 20 and figure 17. Maximum number of paranodes were recorded in 10^{-5} M concentration of naringenin. Uninoculated and naringenin treated plants produced no paranodes. S₁ and S₂ produced paranodular structures even in isolate alone treatment. Maximum number of paranodes were observed in S₁ treated seedlings followed by S₂ and the reference strain at 10^{-5} M concentration. At 10^{-7} M concentration no isolate except S₁ produced any paranodule structure on the rice seedling roots. Inoculation of *A.caulinodans* and naringenin at 3.0ppm caused formation of more nodule like structures than at lower concentrations (Senthil Kumar, 2000).

4.2.2.4 Number of lateral rootlets formed

Addition of naringenin at all concentrations caused increase in number of lateral rootlets formed over the uninoculated control (Table 21 and figure 18).

Table 19: Effect of Naringenin and inoculation of *A. caulinodans* isolates on shoot growth of rice seedlings cv PR-115

Treatments	Shoot Length (cm)			
	Naringenin (M)			
	0	10^{-5}	10^{-6}	10^{-7}
Control	10.0	15.0	13.0	13.0
R3	11.0	16.5	16.0	15.5
R4	12.0	17.0	15.0	13.5
S1	14.5	20.0	19.5	18.0
S2	13.9	19.5	17.0	17.0
ZB-SK-5	13.4	16.0	15.5	15.0

Figure 16

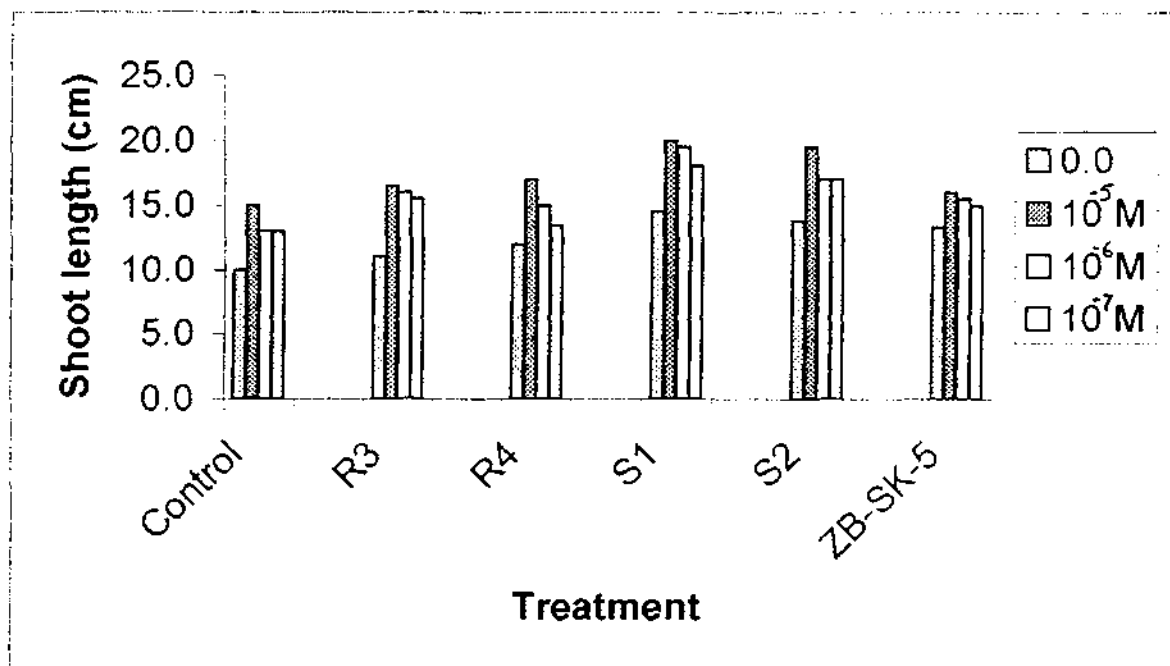


Table 20: Effect of Naringenin and inoculation of *A.caulinodans* isolates on nodule-like structure formation in rice seedlings cv PR-115

Treatments	No. of paranodes formed per plant			
	Naringenin (M)			
	0	10^{-5}	10^{-6}	10^{-7}
Control	-	-	-	-
R3	-	8.0	1.0	-
R4	-	8.5	1.0	-
S1	2.0	14.5	6.0	1.0
S2	1.0	14.0	2.0	-
ZB-SK-5	-	12.0	3.0	-

Figure 17

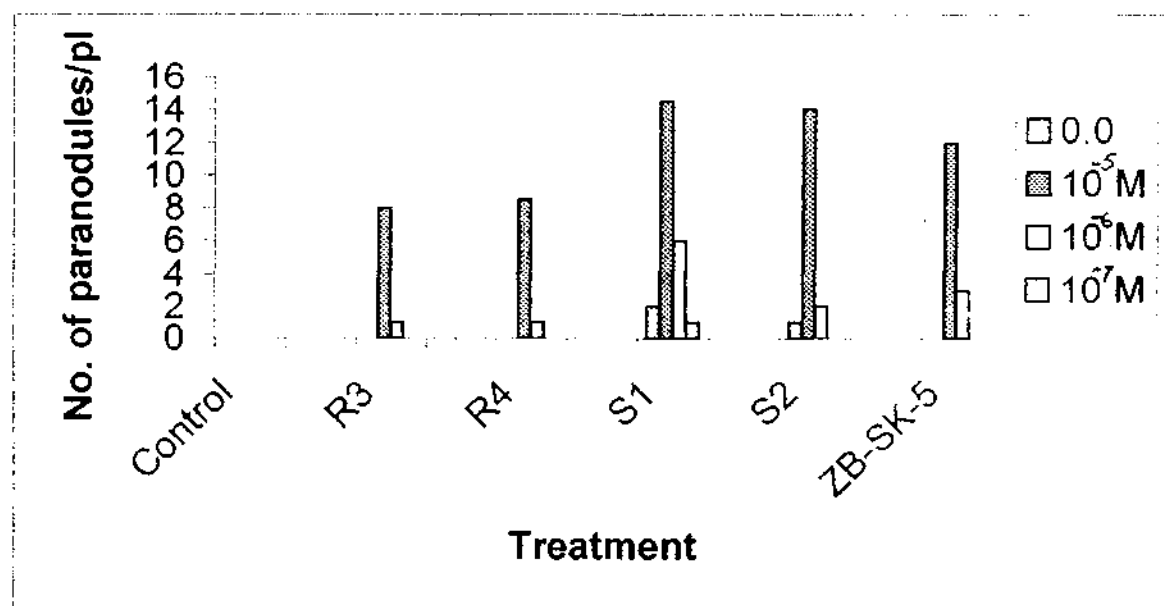
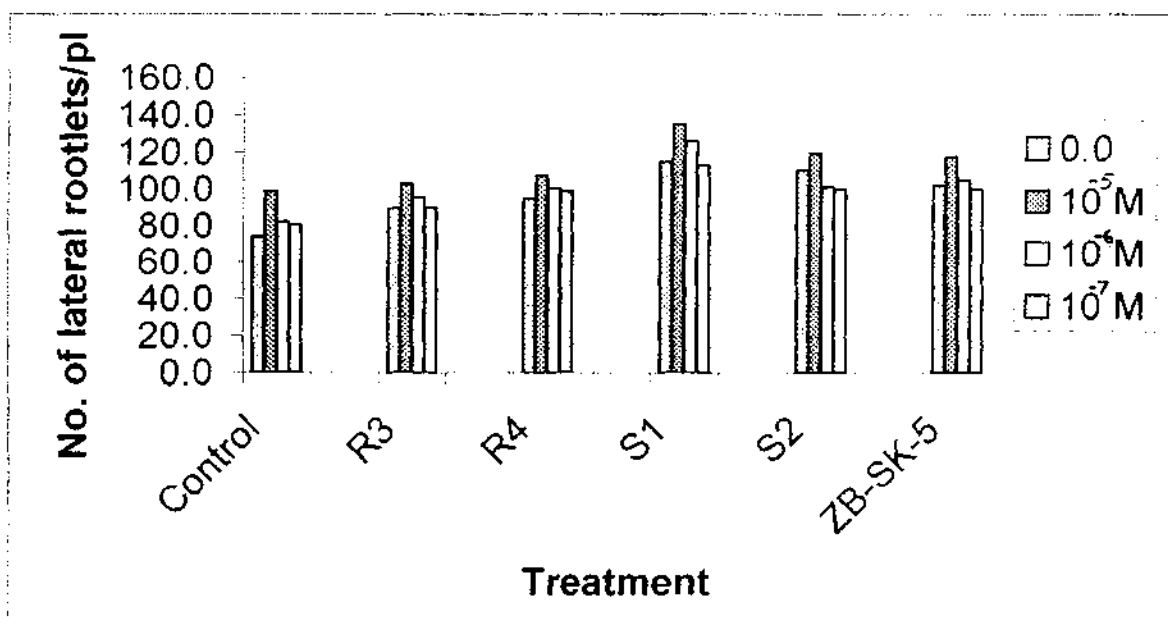


Table 21: Effect of Naringenin and inoculation of *A. caulinodans* isolates on lateral rootlet formation in rice seedlings cv PR-115

Treatments	No. of lateral rootlets per plant			
	Naringenin (M)			
	0	10^5	10^6	10^7
Control	74.0	99.0	82.0	80.5
R3	89.5	103.0	95.5	90.0
R4	95.0	107.5	100.5	98.9
S1	115.0	135.0	126.0	113.0
S2	110.5	119.5	101.3	99.9
ZB-SK-5	102.0	117.5	105.0	100.0

Figure 18



Isolate inoculation led to increase in number of lateral rootlets formed per plant, among which S₁ produced maximum number of lateral rootlets. Similarly S₁ inoculated seedlings recorded maximum lateral rootlet number. A significant increase in number of seminal and adventitious roots in various rice varieties was reported by treatment with naringenin along with inoculation of *A.caulinodans* (Gopalaswamy, 1998) and observed maximum response on inoculation of bacteria 5 days after germination that was irrespective of amount of inoculation used.

4.2.2.5 Fresh and Dry weight

Fresh and dry weights were found to increase of rice seedlings in presence of naringenin in comparison to the uninoculated control (Table 22 and 23 and figures 19 and 20). Inoculation of azorhizobial isolates alone increased this parameter in rice. S₁ produced maximum fresh and dry weight followed by S₂ and R₄. 10⁻⁵M concentration showed best results among all isolates in all three concentrations. Buvana (2001) observed an increase in shoot length, total fresh biomass and dry weight in rice and maize seedlings with *A.caulinodans*, naringenin, hydrolytic enzyme treatment and growth regulator.

4.2.2.6 Nitrogenase activity

The results are presented in Table 24 and figure 21. No nitrogenase activity was recorded in uninoculated naringenin treated control. Likewise nitrogenase activity was not observed in any of the isolate as well as reference strain inoculated seedlings. Maximum nitrogenase activity was recorded in 10⁻⁵M concentration of naringenin followed by 10⁻⁶ M and 10⁻⁷ M concentrations. Among

Table 22: Effect of Naringenin and inoculation of *A.caulinodans* isolates on fresh weight of rice seedlings cv PR-115

Treatment	Fresh Weight (g/pl)			
	Naringenin (M)			
	0	10^{-5}	10^{-6}	10^{-7}
Control	0.045	0.052	0.047	0.048
R3	0.051	0.060	0.052	0.051
R4	0.052	0.060	0.052	0.051
S1	0.055	0.064	0.058	0.054
S2	0.053	0.062	0.056	0.052
ZB-SK-5	0.051	0.061	0.053	0.049

Figure 19

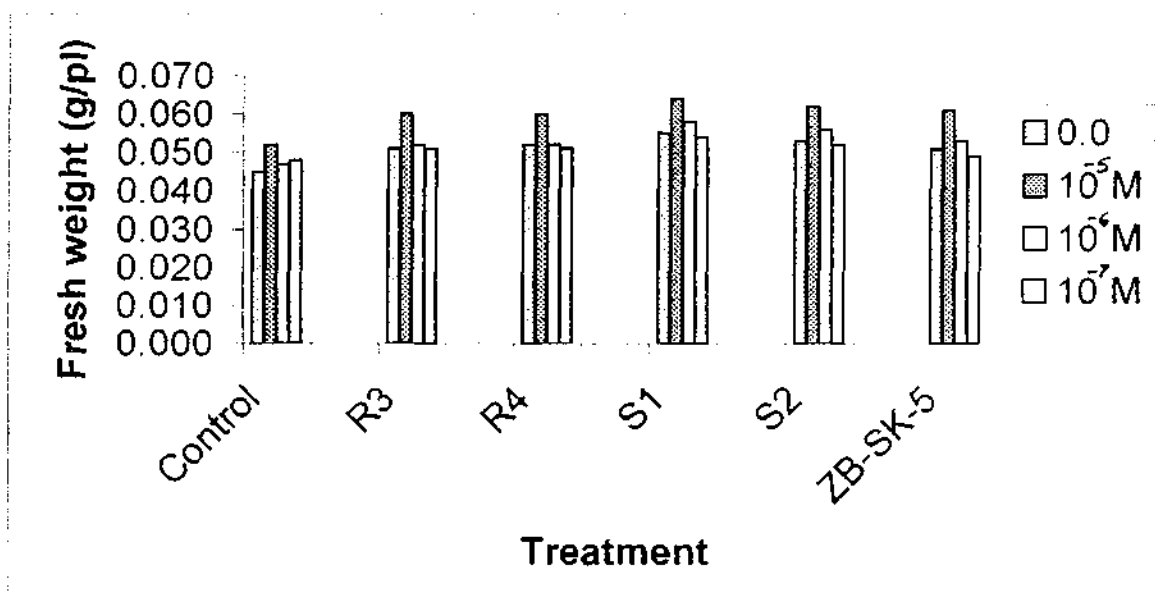


Table 23: Effect of Naringenin and inoculation of *A. caulinodans* isolates on dry weight of rice seedlings cv PR-115

Treatments	Dry Weight (mg/plant)			
	Naringenin (M)			
	0	10^{-5}	10^{-6}	10^{-7}
Control	4.0	5.0	4.3	4.2
R3	4.5	5.6	5.0	4.6
R4	4.7	5.7	5.0	4.8
S1	5.1	6.0	5.3	5.0
S2	4.8	6.0	5.1	4.9
ZB-SK-5	4.6	5.9	5.0	4.5

Figure 20

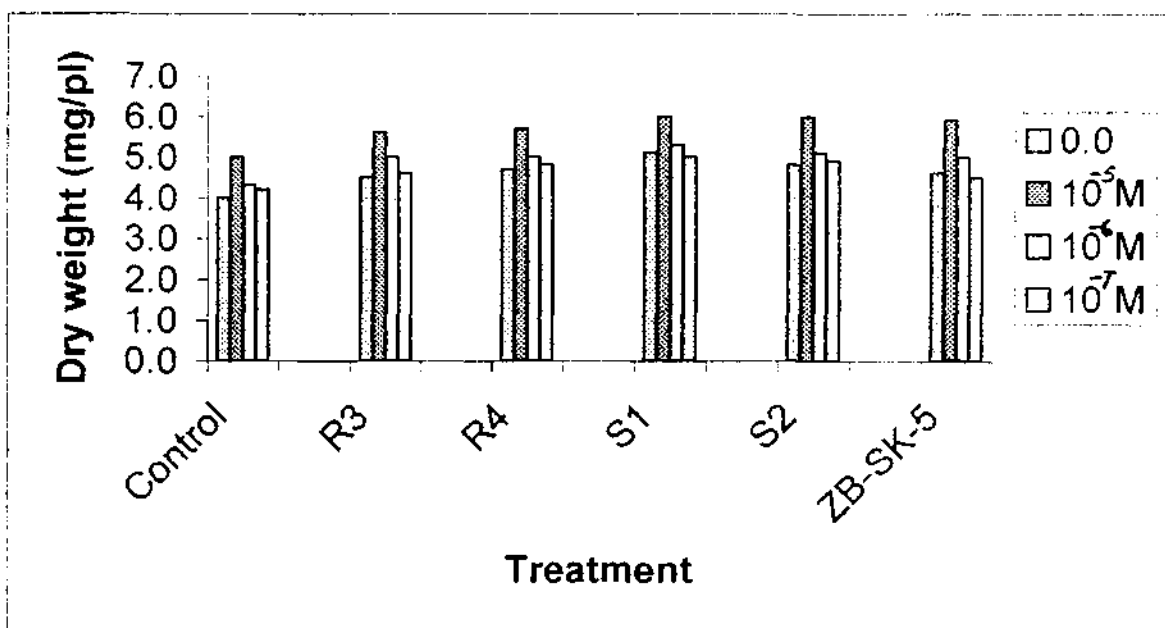


Table 24: Effect of Naringenin and inoculation of *A. caulinodans* isolates on nitrogenase activity of rice seedlings cv PR-115

Treatments	Nitrogenase activity(nM/pl/20h)			
	Naringenin (M)			
	0	10^{-5}	10^{-6}	
Control	-	-	-	-
R3	-	3.86	3.00	2.3
R4	-	6.23	5.50	3.7
S1	3.6	7.45	6.80	5.3
S2	2.0	7.00	6.30	4.7
ZB-SK-5	-	7.30	6.50	5.1

Figure 21

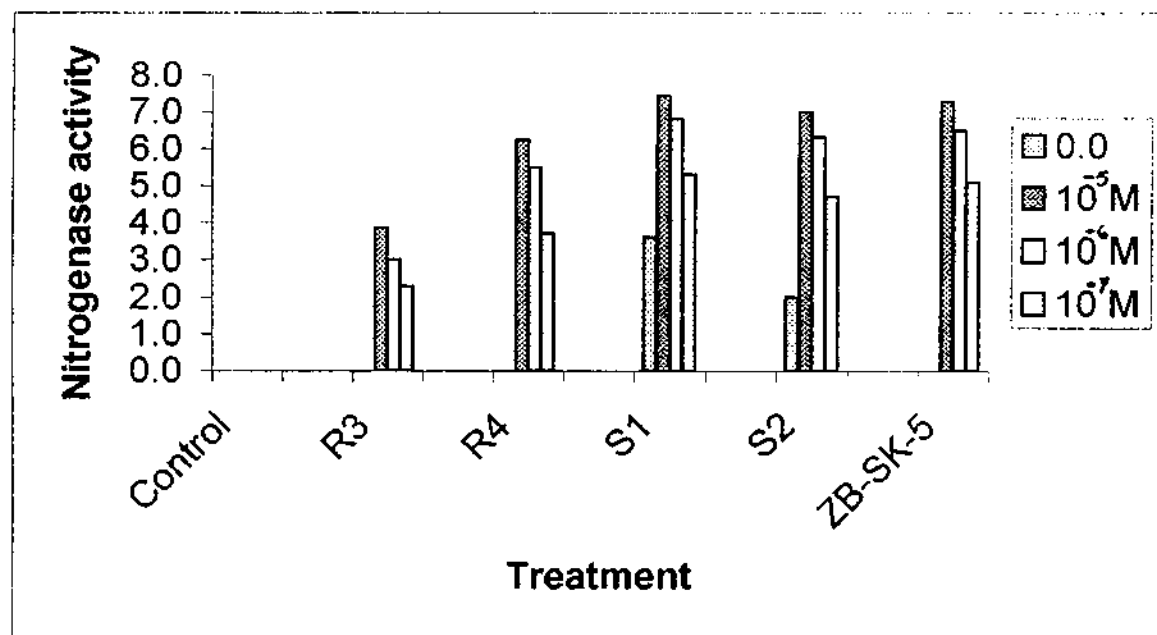


Plate 11: Effect of inoculation of *A. caulinodans* isolate in presence of Naringenin (10^{-5} M)

Plate 12: Stained microtomic section of rice roots treated with naringenin and *A. caulinodans* isolates

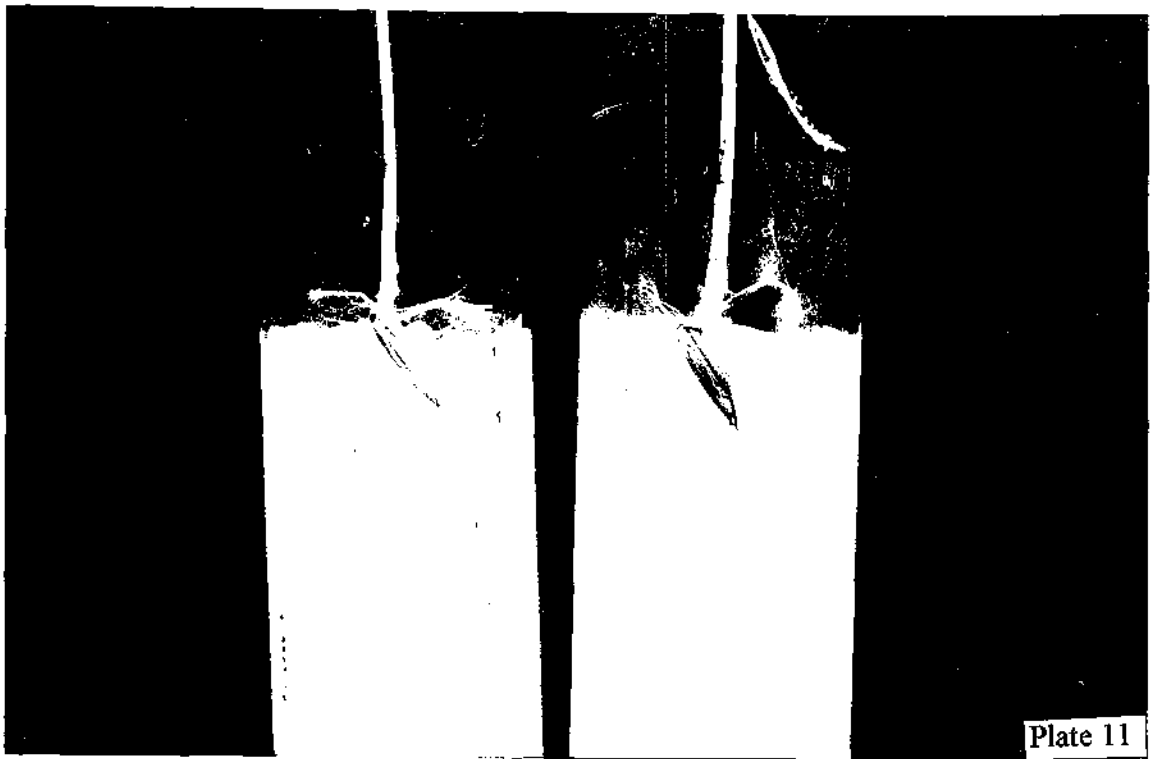


Plate 11

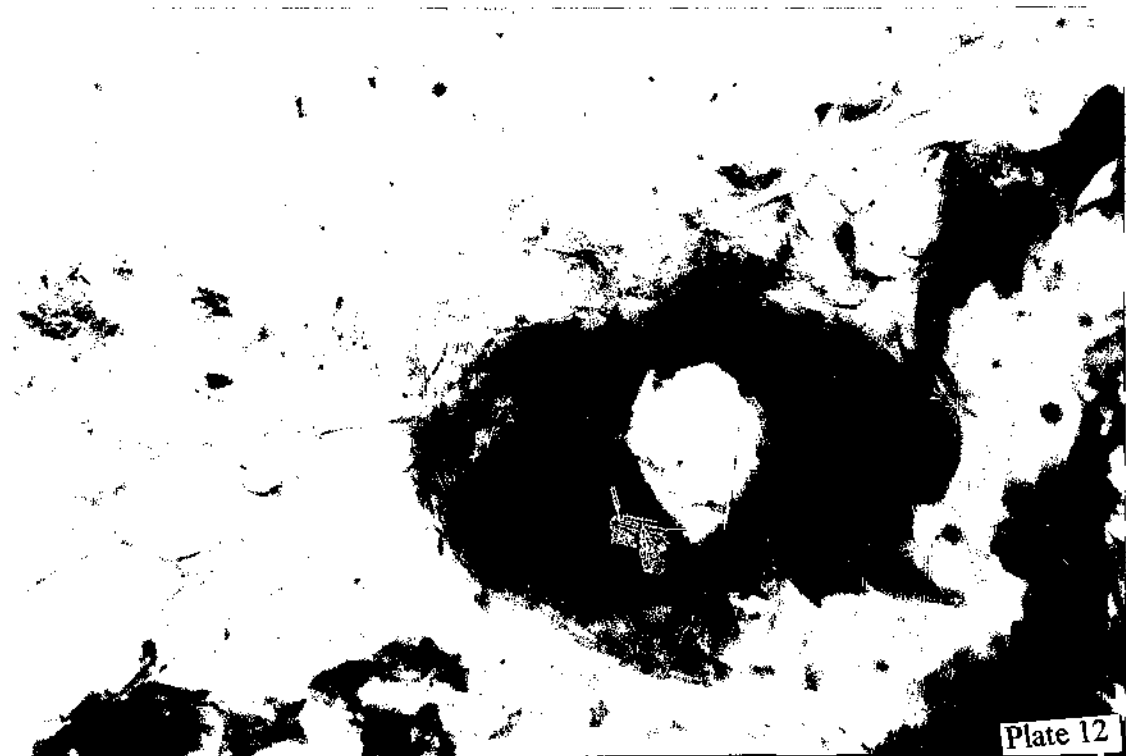


Plate 12

all isolates S₁ performed best at all concentrations used. Senthil Kumar (2000) reported nitrogenase activity in rice and maize seedlings treated with 7.0 ppm NAA, 3.0 ppm Naringenin and cellulase + pectinase enzyme mixture.

4.2.2.7 Histological studies

The Naringenin (10^{-5} M) and isolate S₁ treated roots showed increased surface accumulation of *A.caulinodans* cells with certain cells entering the root cortex present intercellularly (Plate 12). Thus the use of signal compounds induces the inward encroachment of the azorhizobia as some signal compounds act as polar auxin transporters (Jacobs and Rubery, 1988) and so increase the accumulation of auxins at a particular site such as roots. This causes increase in number of lateral rootlets and thus the crack entry.

At all concentrations of naringenin results recorded showed an increase in root and shoot length, fresh and dry weight, paranodule and lateral rootlet number and nitrogenase activity. Naringenin at 10^{-6} M concentration produced best results with S₁ being the best performer amongst all.

4.2.2(b) Effect of Acetosyringone on rice seedlings PR-115

4.2.2.8 Root growth

The results are presented in Table 25 and figure 22. Root length was recorded to decrease in isolates inoculated seedlings in comparison to uninoculated control (Plate 13 and 14). *A.caulinodans* ZB-SK-5 produced maximum decrease in root length in culture alone treated seedlings. Similarly a sharp decrease in root length was observed at both concentrations with all isolates as well as in the uninoculated control. Maximum decrease in root length

Plate 13: Effect of inoculation of *A. caulinodans* isolates on root length of rice cv PR-115 in presence of Acetosyringone (266 ppm)

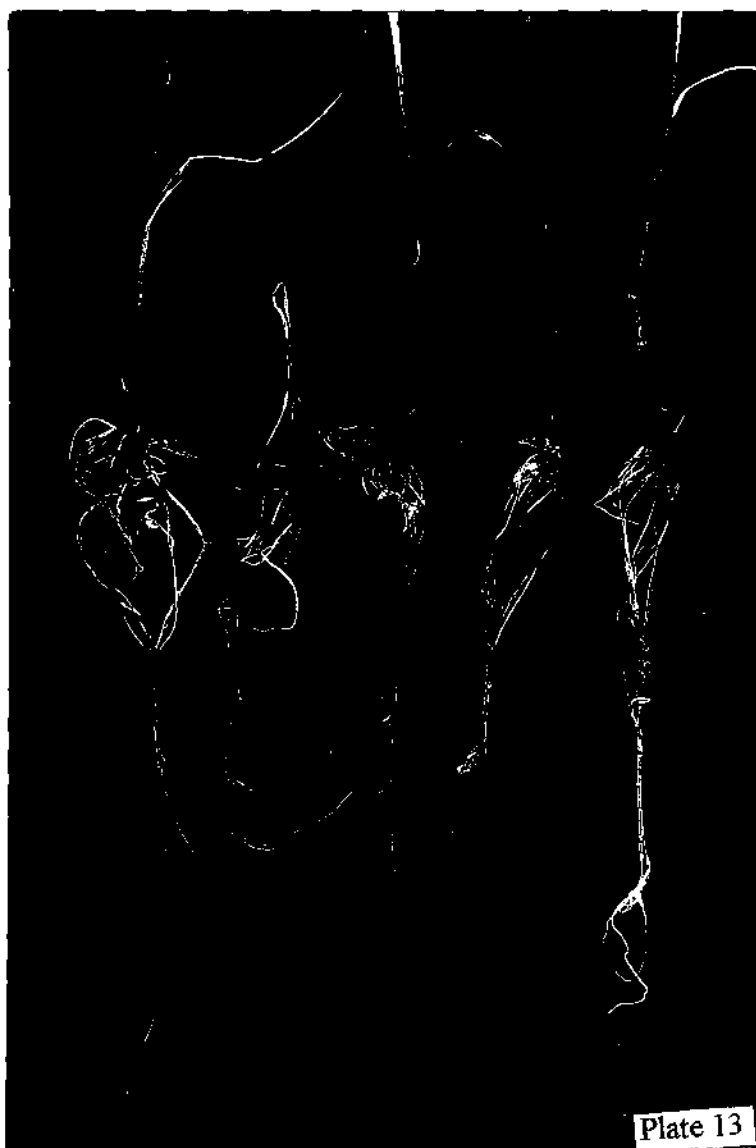


Plate 13

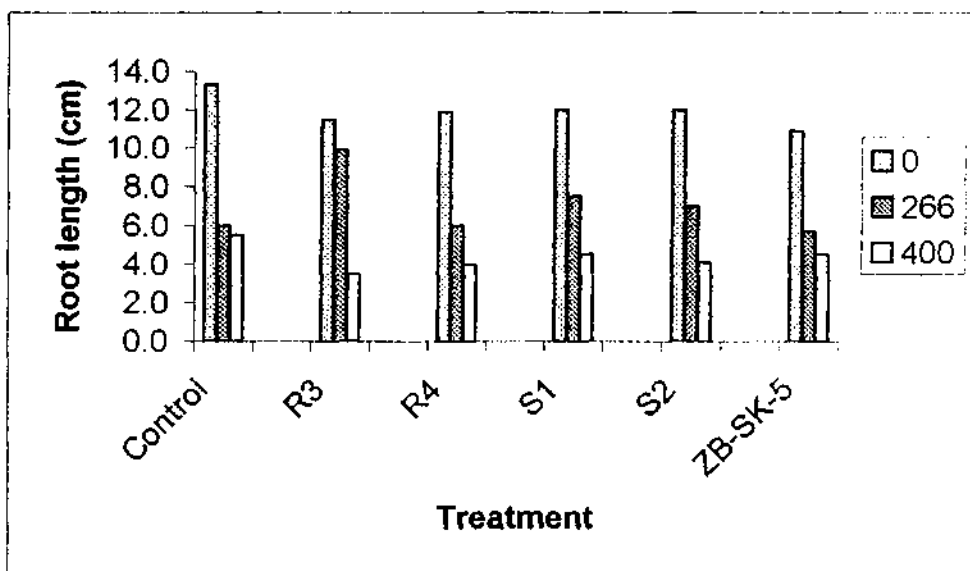
Plate 14: Effect of inoculation of *A. caulinodans* isolates on root length of rice cv PR-115
in presence of Acetosyringone (400 ppm)



Table 25: Effect of Acetosyringone and inoculation of *A.caulinodans* isolates on root growth of rice seedlings cv PR-115

Treatments	Root Length (cm)		
	Acetosyringone (ppm)		
	0	266	400
Control	13.3	6.0	5.5
R3	11.5	9.90	3.50
R4	11.9	6.00	4.00
S1	12.0	7.50	4.50
S2	12.0	7.00	4.10
ZB-SK-5	10.9	5.70	4.50

Figure 22



was observed for R₃ treated seedlings at 400 ppm concentration followed by R₄. The reduction in root length indicates that the signal compounds as acetosyringone causes the formation of phytohormones as 2,4-D or its production from the bacteria inoculated.

4.2.2.9 Shoot growth

The results presented in Table 26 and figure 23 that clearly showed a decrease in shoot length in Acetosyringone treated plants at both concentrations. S₁ inoculated seedlings produced maximum shoot height followed by the reference strain. At 400 ppm concentration seedlings gave better results over 266 ppm concentration. The increase in shoot length could be produced by the increase in the phytohormone concentration due to the inoculation by *A. caulnodans* isolates and the compound i.e. Acetosyringone.

4.2.2.10 Paranodule formation

Acetosyringone was observed to induce paranodulation in rice. The results showed that the uninoculated acetosyringone treated seedlings produced no such structures in rice (Table 27 and figure 24). All isolate alone treatments showed paranodulation except R₃. Treatment with the chemical resulted in paranodule formation on rice roots. R₄ treated plants recorded maximum number of paranodes at both concentrations followed by S₁. Though no report is there regarding the nodulation in rice by acetosyringone but Bolton *et al* (1986) have reported that acetosyringone shows the greatest inducing activity for *Agrobacterium tumefaciens vir* genes that cause tumour formation in the infected host.

Table 26: Effect of Acetosyringone and inoculation of *A. caulinodans* isolates on shoot growth of rice seedlings cv PR-115

Treatments	Shoot Length (cm)		
	Acetosyringone (ppm)		
	0	266	400
Control	13.5	12.3	12.5
R3	14.0	12.5	13.0
R4	14.3	12.5	13.5
S1	15.5	12.0	14.1
S2	14.9	12.9	13.7
ZB-SK-5	15.1	12.5	13.0

Figure 23

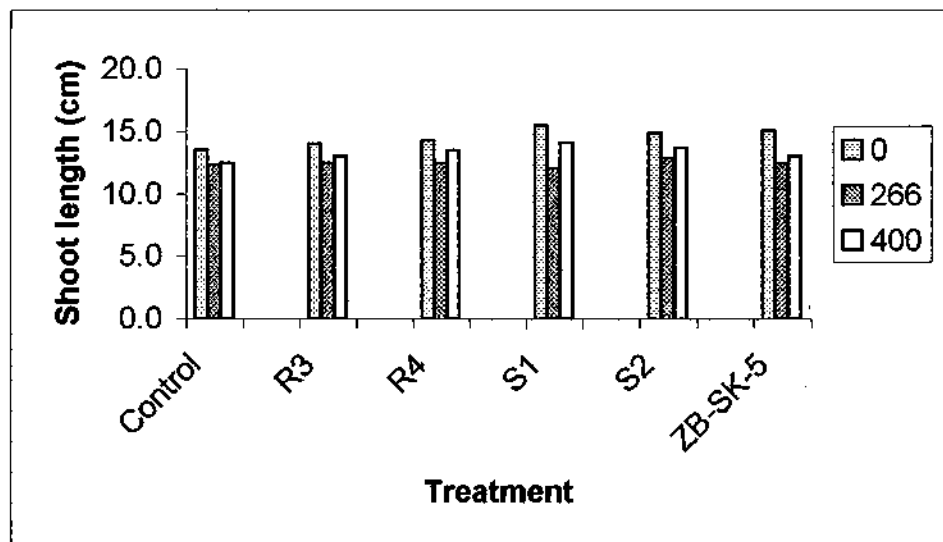
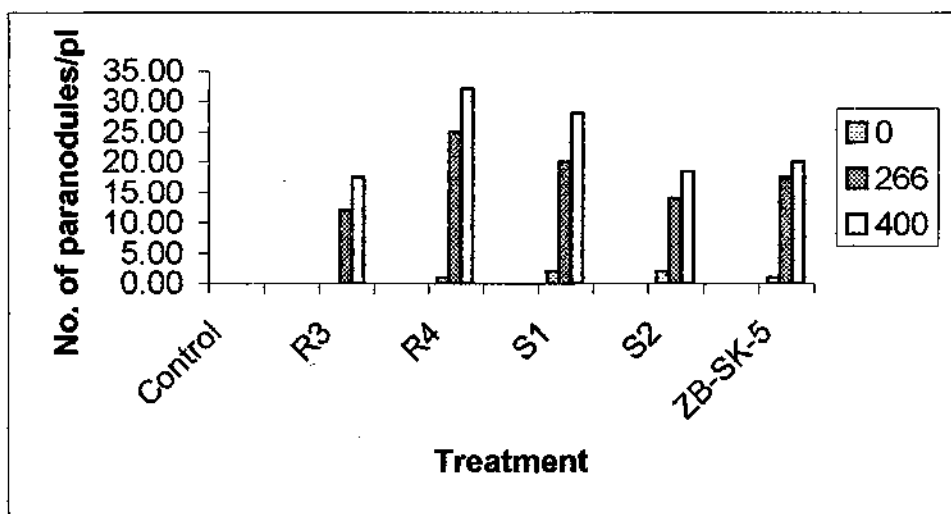


Table 27: Effect of Acetosyringone and inoculation of *A. caulinodans* isolates on nodule-like structure formation in rice seedlings cv PR-115

Treatment	No. of paranodes per plant		
	Acetosyringone (ppm)		
	0	266	400
Control	-	-	-
R3	-	12.0	17.5
R4	1	25.0	32.0
S1	2	20.0	28.0
S2	2	14.0	18.5
ZB-SK-5	1	17.5	20.0

Figure 24



4.2.2.11 Number of lateral rootlets formed

The number of lateral rootlets was recorded to decrease in presence of the chemical in comparison to the untreated control (Table 28 and figure 25). Inoculation of *A.caulinodans* isolates resulted in decrease in number of lateral rootlets formed per plant (Plate 15). Maximum number of lateral rootlets were recorded in S₁ followed by S₂. S₁ treated seedlings produced maximum number of lateral rootlets at 266 ppm concentration while R₄ at 400 ppm gave highest number of rootlets. The number of lateral rootlets increased due to the increase in the amount of the growth hormones either secreted by the infecting azorhizobia or due to the increased transportation of such compounds as signal compounds function as endogenous polar auxin transporters (Jacobs and Rubery, 1988).

4.2.2.12 Fresh and dry weight

An increase in fresh and dry weight was recorded in isolate inoculated seedlings over the uninoculated control (Table 29 and 30 and figures 26 and 27). S₁ inoculated plants showed maximum fresh weight production in isolate alone treatments. R₄ treated plants gave maximum fresh and dry weight at both concentrations. The fresh and dry weight increase may have occurred due the increase in the auxins available.

4.2.2.13 Nitrogenase activity

The results presented in Table 31 and figure 28 revealed no nitrogenase activity in uninoculated control plants. Among the isolates R₄ produced maximum

Table 28: Effect of Acetosyringone and inoculation of *A.caulinodans* isolates on lateral rootlet formation in rice seedlings cv PR-115

Treatments	No. of lateral rootlets per plant		
	Acetosyringone (ppm)		
	0	266	400
Control	43.0	30.0	24.0
R3	71.0	54.0	39.0
R4	69.3	62.0	58.0
S1	80.0	64.0	52.0
S2	78.5	59.0	45.0
ZB-SK-5	70.0	76.0	62.0

Figure 25

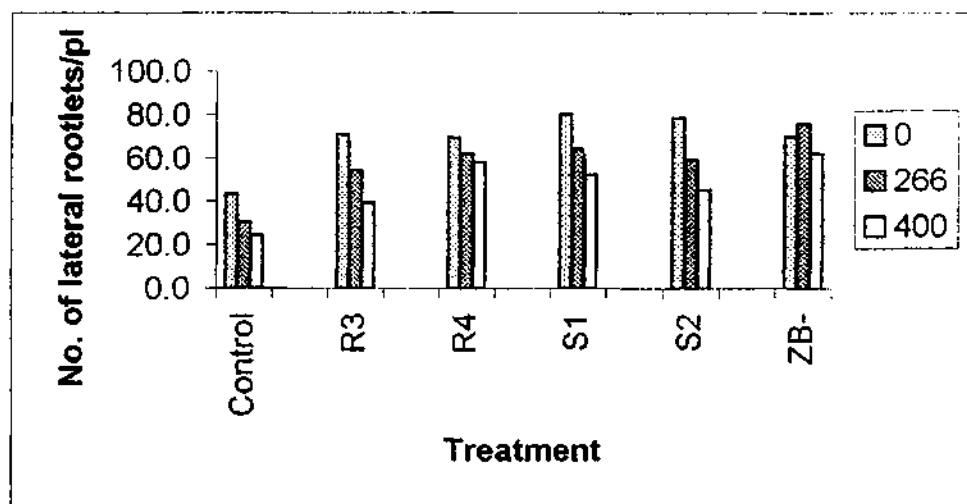


Table 29: Effect of Acetosyringone and inoculation of *A. caulinodan* isolates on fresh weight of rice seedlings cv PR-115

Treatments	Fresh Weight (g/plant)		
	Acetosyringone (ppm)		
	0	266	400
Control	0.049	0.053	0.065
R3	0.051	0.050	0.053
R4	0.050	0.059	0.061
S1	0.054	0.055	0.058
S2	0.052	0.054	0.059
ZB-SK-5	0.050	0.055	0.057

Figure 26

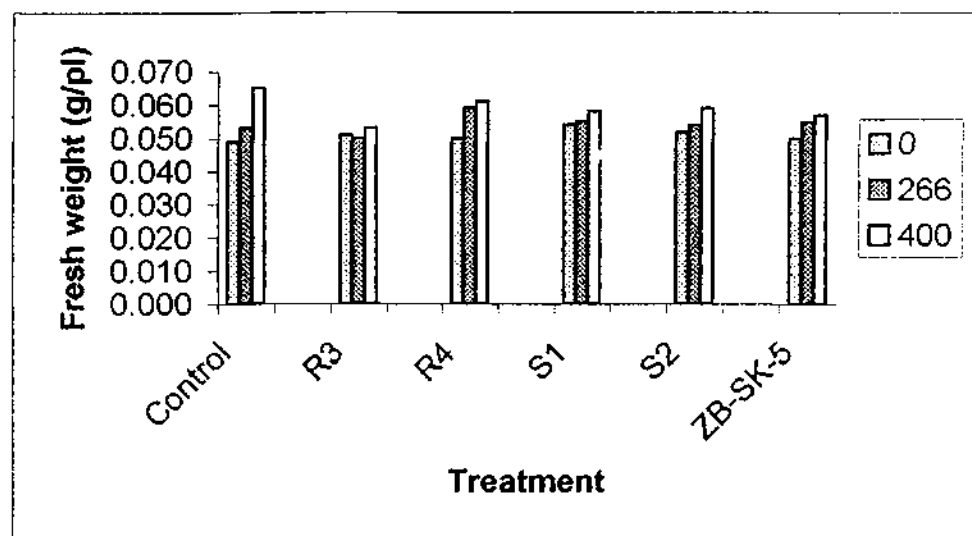


Table 30: Effect of Acetosyringone and inoculation of *A. caulinodan* isolates on dry weight of rice seedlings cv PR-115

Treatments	Dry Weight (mg/plant)		
	Acetosyringone (ppm)		
	0	266	400
Control	4.0	5.0	6.0
R3	4.5	5.1	5.0
R4	5.1	9.0	8.0
S1	4.8	7.0	6.1
S2	4.5	6.5	6.0
ZB-SK-5	4.4	8.0	6.0

Figure 27

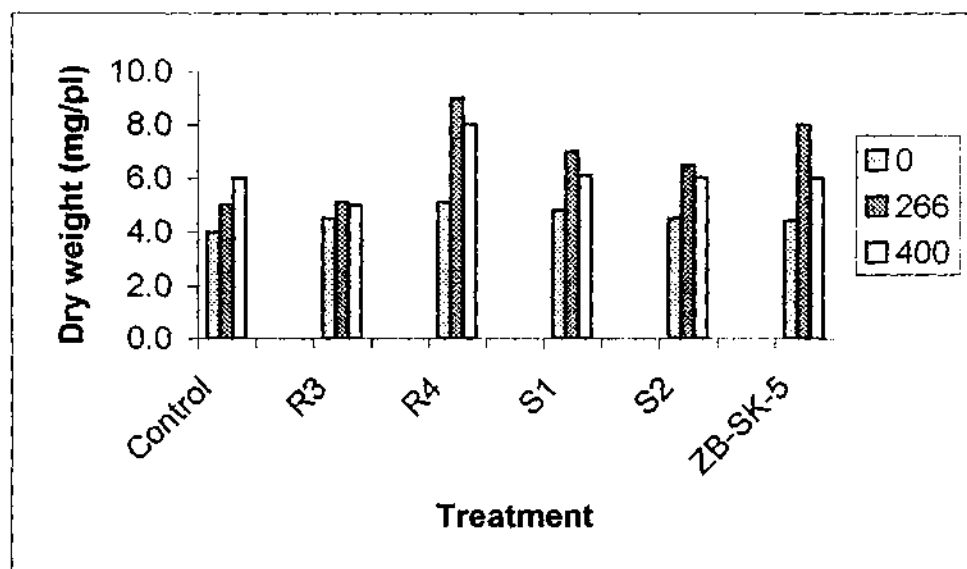


Table 31: Effect of Acetosyringone and inoculation of *A.caulinodans* isolates on root nitrogenase activity of rice seedlings cv PR-115

Treatments	Nitrogenase activity (nM/pl/20h)		
	Acetosyringone (ppm)		
	0	266	400
Control	-	-	-
R3	-	6.0	12.0
R4	-	11.0	29.9
S1	-	6.3	12.6
S2	-	6.1	12.0
ZB-SK-5	-	6.3	11.0

Figure 28

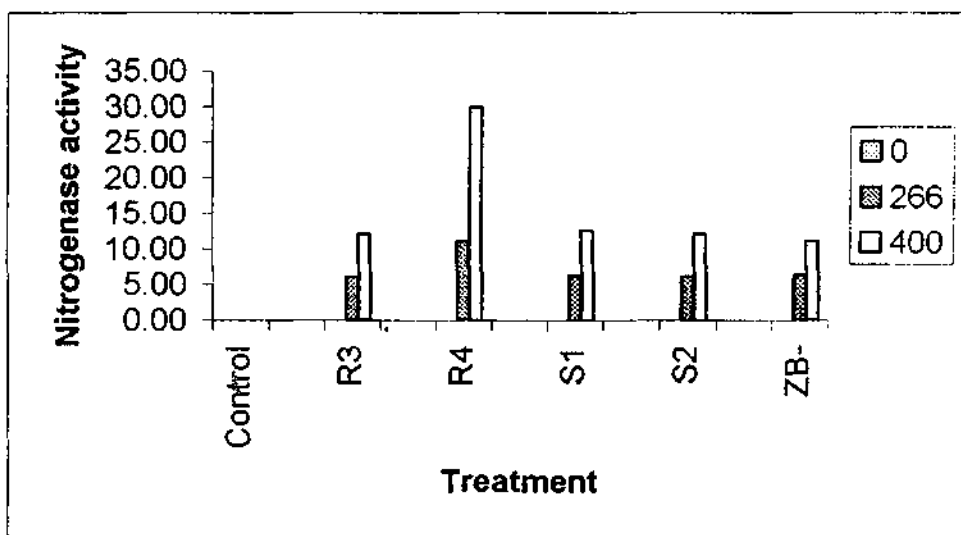


Plate 15: Close up view of the nodule like structure in rice root treated with Acetosyringone and inoculation of *A. caulinodans* isolate R₄

Plate 16: Stained microtomic section of rice root inoculated with *A. caulinodans* isolate R₄ in presence of Acetosyringone

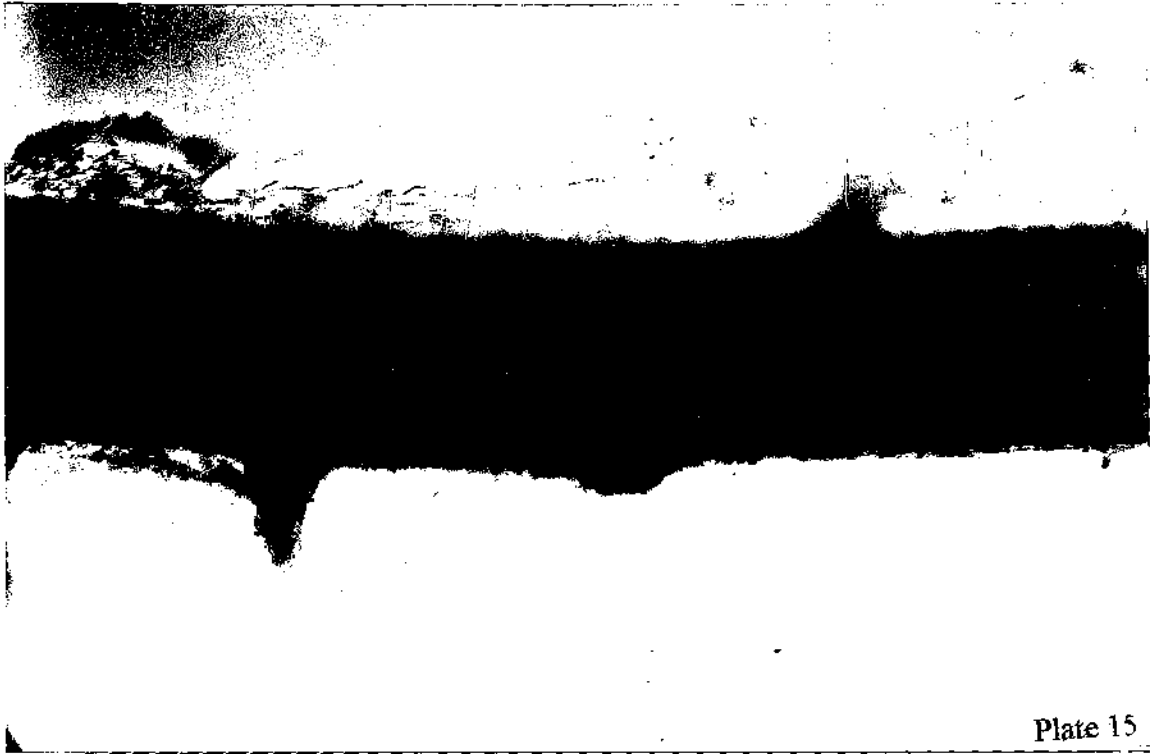


Plate 15



Plate 16

Figure 29: Effect of inoculation of azorhizobial isolates and various chemical root length of rice cv PR-115

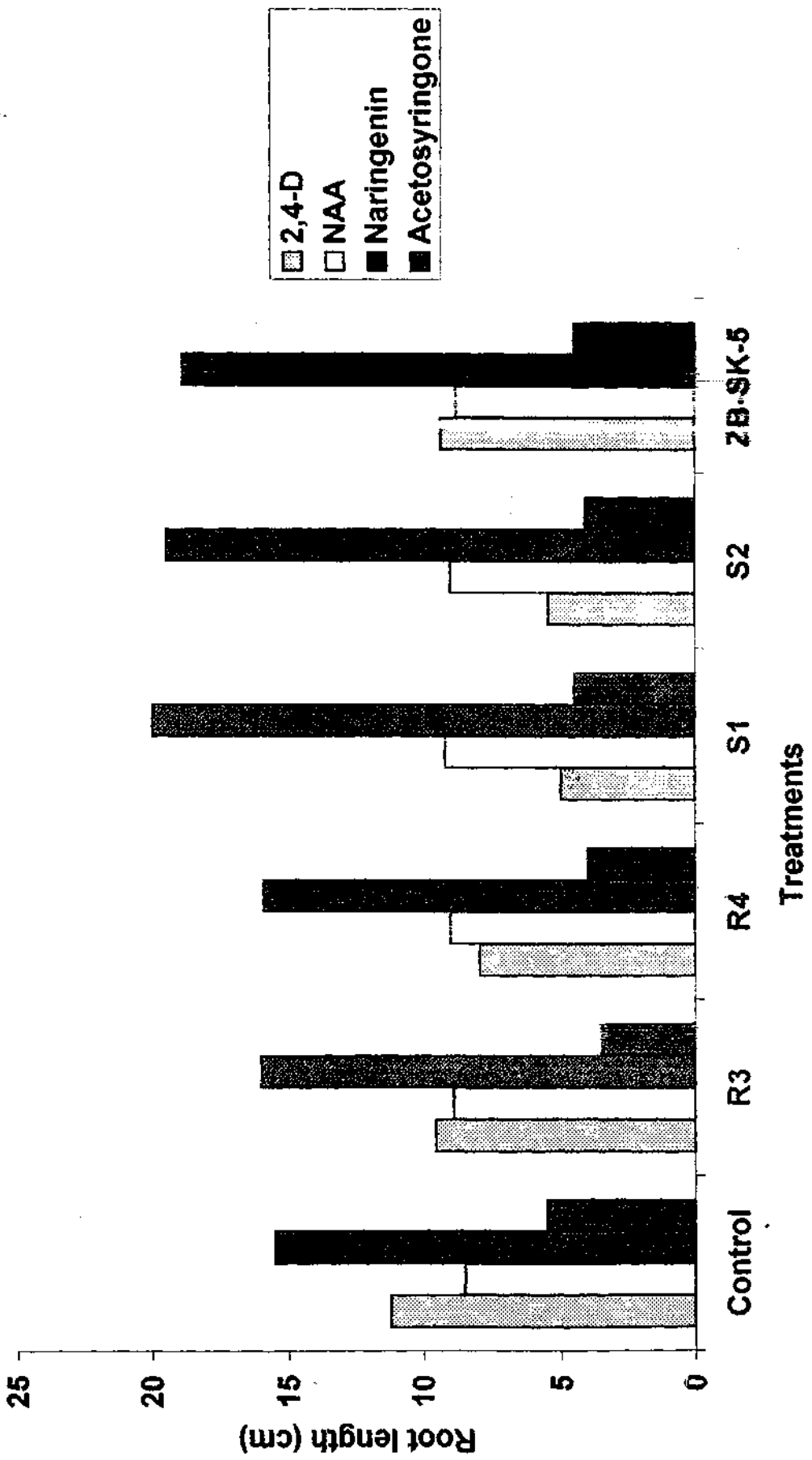


Figure 30: Effect of inoculation of azorhizobial isolates and various chemicals on shoot length of rice cv PR-115.

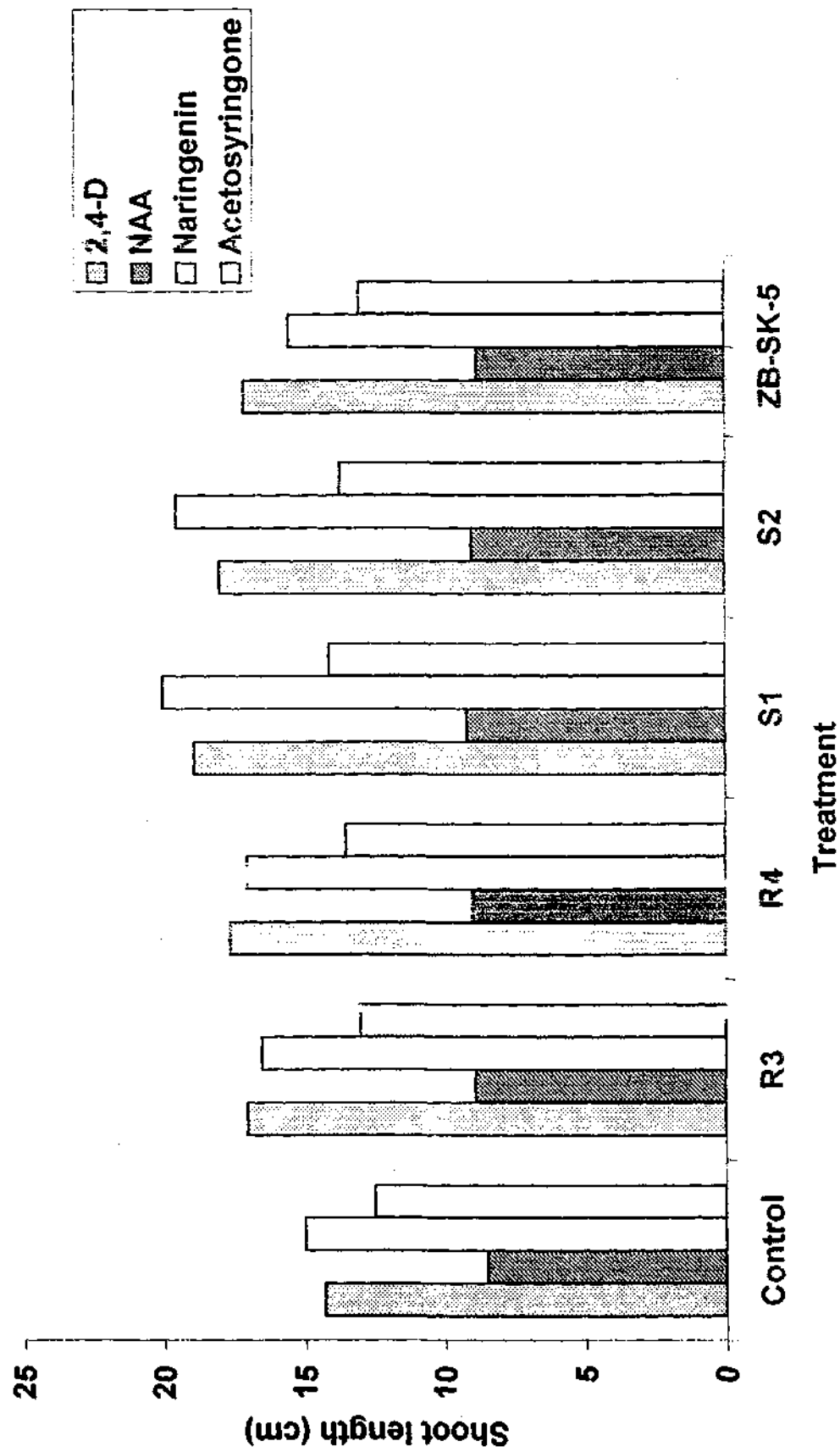


Figure 31: Effect of inoculation of azorhizobial isolates and various chemicals on paranodule formation in rice cv PR-115.

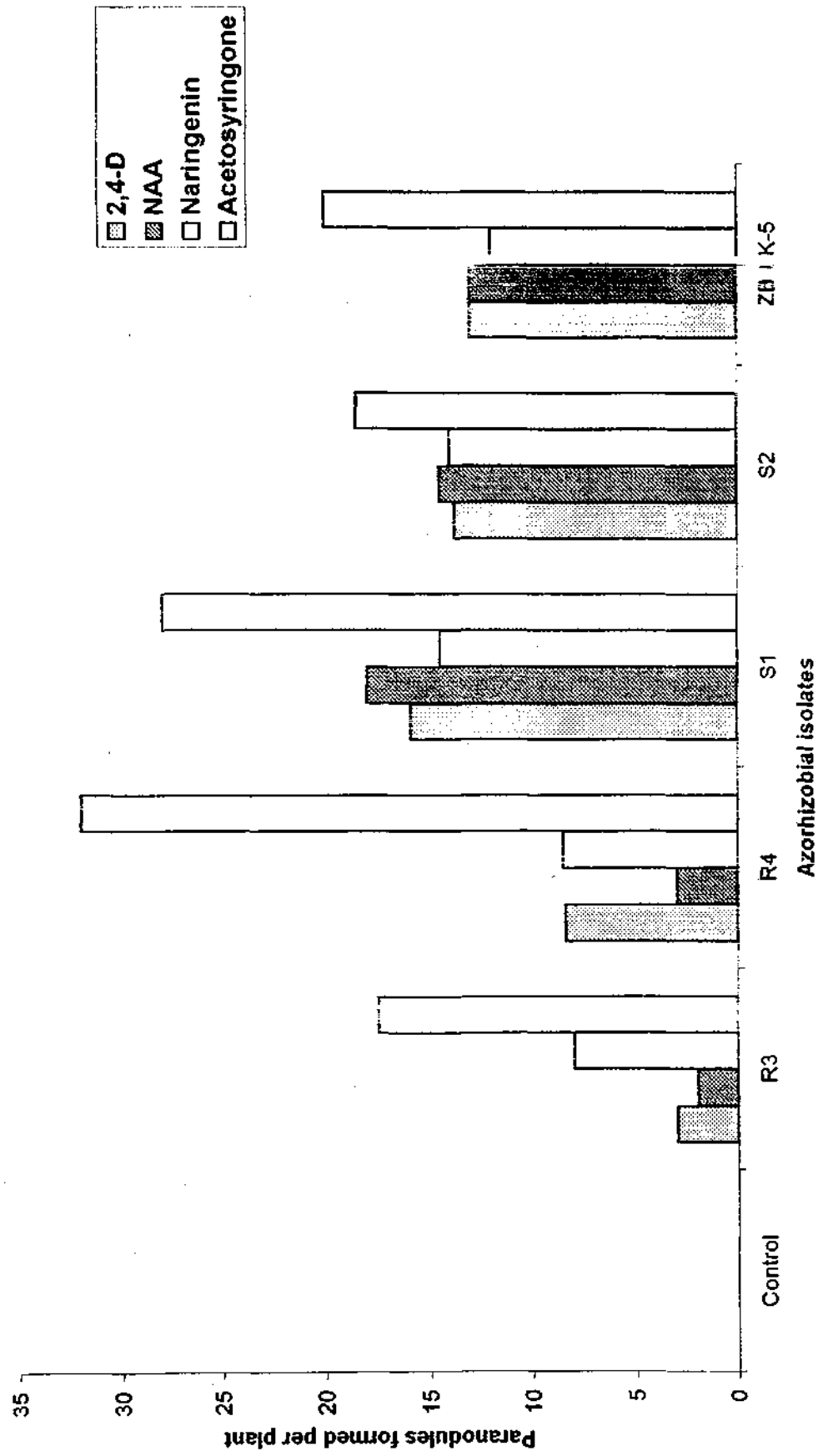


Figure 32: Effect of inoculation of azorhizobial isolates and various chemicals on lateral rootlet formation in rice cv PR-115.

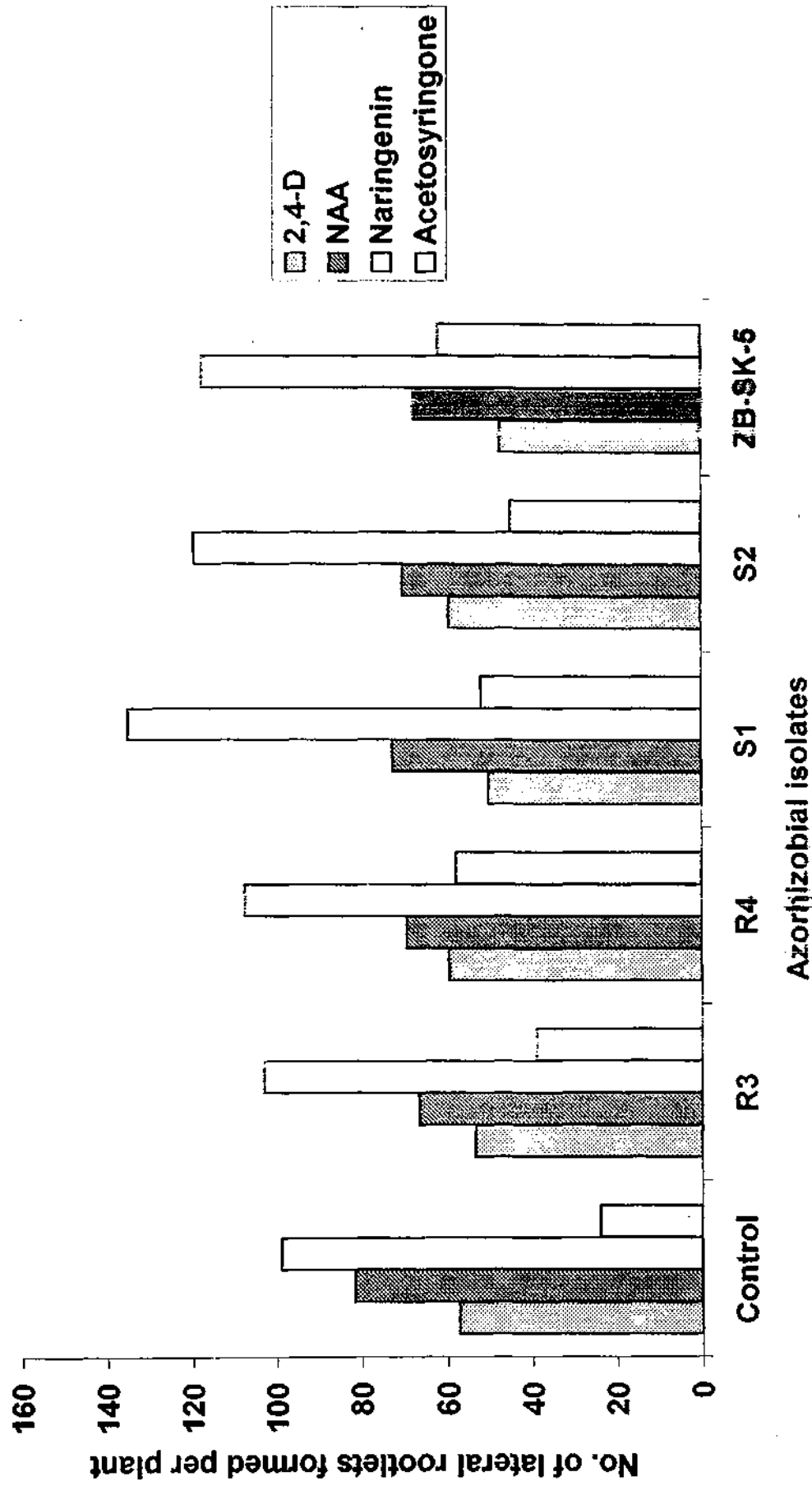


Figure 33: Effect of inoculation of azorhizobial isolates and various chemicals on fresh weight of rice cv PR-115.

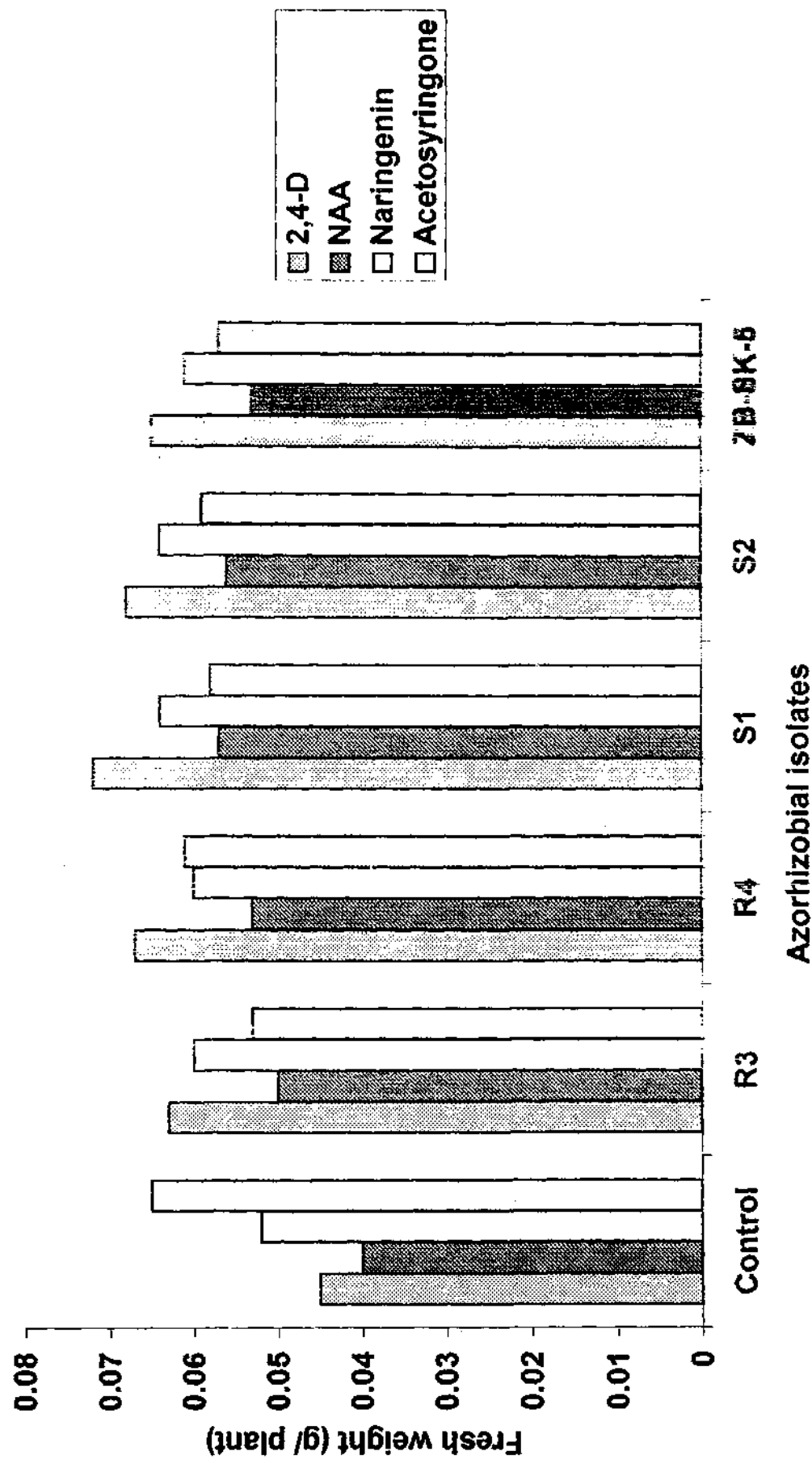


Figure 34: Effect of inoculation of azorhizobial isolates and various chemicals on dry weight of rice cv PR-115.

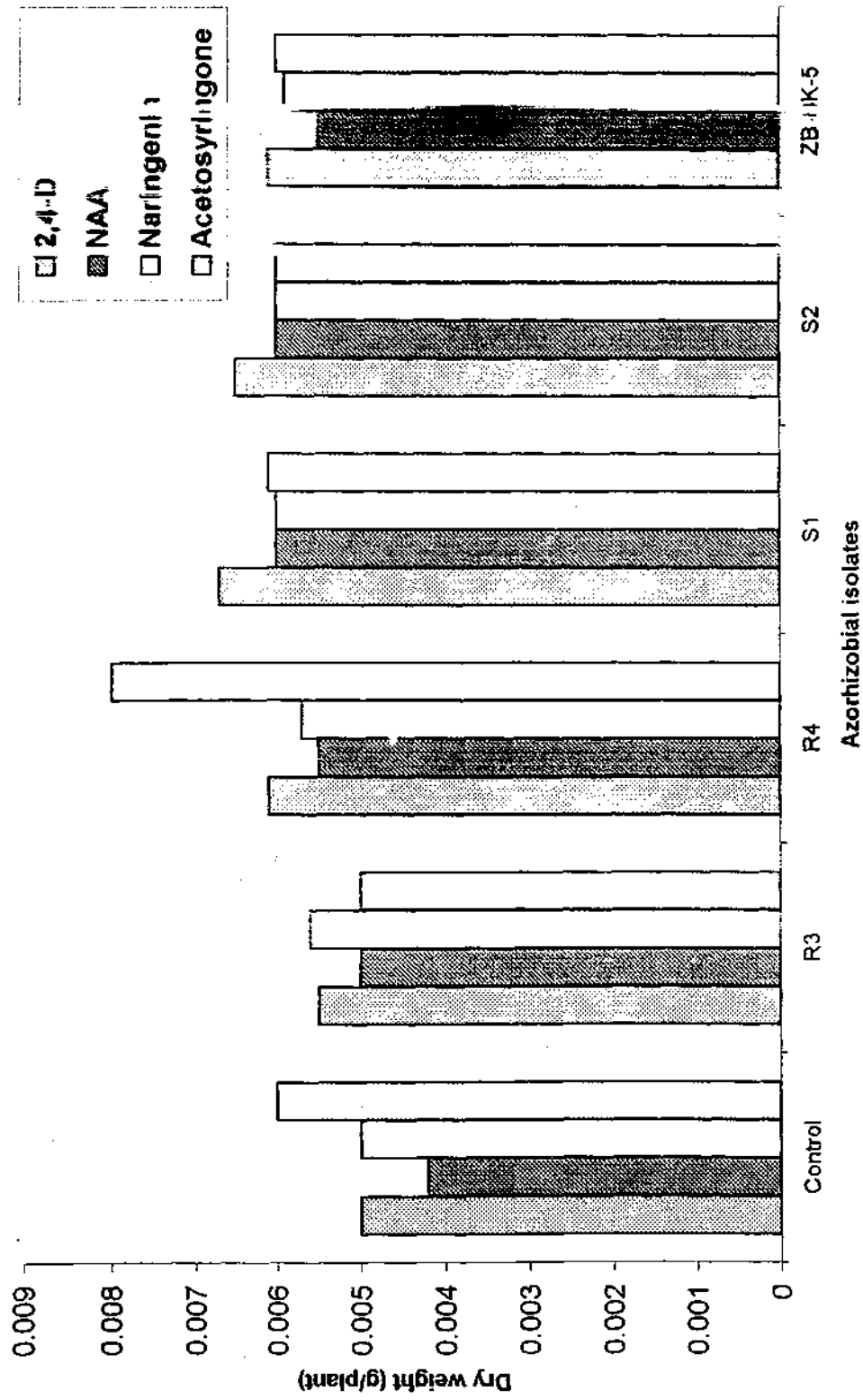
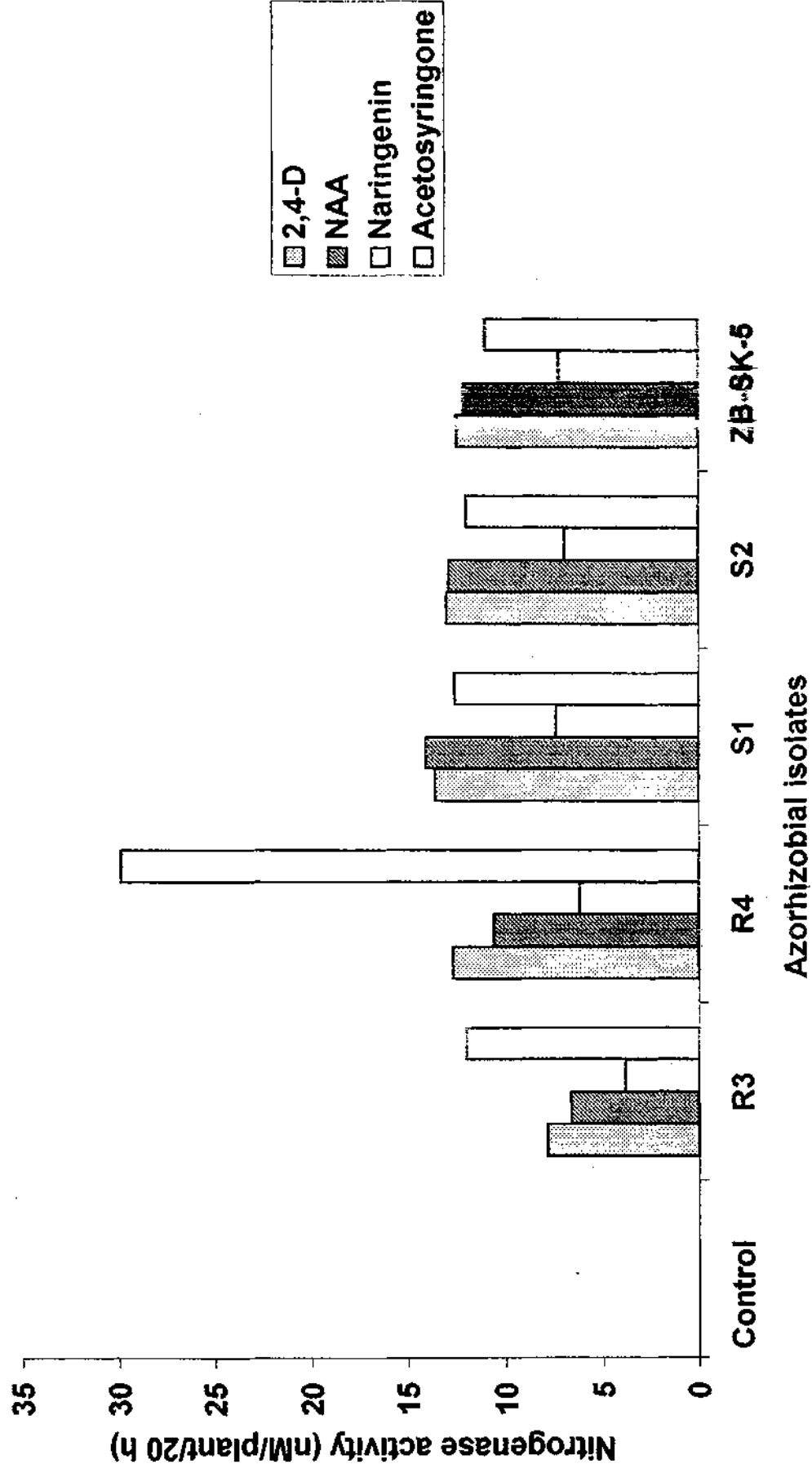


Figure 35: Effect of inoculation of azorhizobial isolates and various chemicals on nitrogenase activity of rice cv PR-115.



nitrogenase activity at both concentrations. An increase in concentration of acetosyringone resulted in increase in nitrogenase activity of all isolates.

4.2.2.14 Histological studies

The microtomic sections of the rice roots revealed the presence of azorhizobia on surface of the rice roots with the R₄ and Acetosyringone at 400 ppm treated roots showed increased accumulation on surface and below the rice root epidermis (Plate 16). Though no reports are there regarding the use of acetosyringone in rice nodulation but Stone *et al* (2000) have reported the colonization of *A.caulinodans* ORS571 in roots of *Arabidopsis thaliana*.

Acetosyringone addition at both concentrations resulted in decrease in root length while an increase in shoot length, lateral rootlet and paranodule number, fresh and dry weight and nitrogenase activity. At a concentration of 400 ppm acetosyringone gave better results with R₄ being the best performer.

4.3 Effect of inoculation of *Azorhizobium caulinodans* isolates along with the reference culture on *Sesbania rostrata*.

The six isolates obtained from root and stem nodules of *S.rostrata* were used for both pot and field trails to study their effect on plant height, root and stem nodule formation, nitrogenase activity and the competitiveness of inoculated isolates.

4.3.1(a) Pot Experiment

4.3.1.1 Plant Height

The plant height varied from 31.06-71.73 cm (Table 32 and figure 36). Most of the isolates worked better over control. S₂ gave best performance among all isolates and was found to be significantly better over S₁. Among the root isolates R₁ gave the maximum plant height followed by R₂. All roots isolates and the reference culture were observed to be statistically at par with each other and significant over the control. Kannaiyan and Kalidurai (1995) have reported the increase in the height of the azorhizobia inoculated *S.rostrata* plants at the 60th day after sowing.

4.3.1.2 Stem fresh biomass

The stem fresh biomass ranged from 1.51–3.50 g (Table 33 and figure 37). All isolates except R₃ and R₄ (which were statistically at par with the control) gave significantly more fresh biomass of plants over the control. Among the root isolates R₁ and R₂ were found to be statistically at par with *A.caulinodans* ZB-SK-5 while the other two i.e. R₃ and R₄ were significantly lesser than the reference strain. Both the stem isolates were significantly better over the reference strain. The S₂ performed best among all isolates. The *S.rostrata* produces significant amount of biomass (Dreyfus *et al*, 1985) which was found to increase upon Azorhizobial isolates inoculation due to increased nitrogen available to the plant via nitrogen fixation done by the nodules formed.

Plate 17: *S.rostrata* plants seed inoculated with *A.caulinodans* grown under pot conditions

Plate 18: *A.caulinodans* inoculated *S.rostrata* plants grown under field conditions

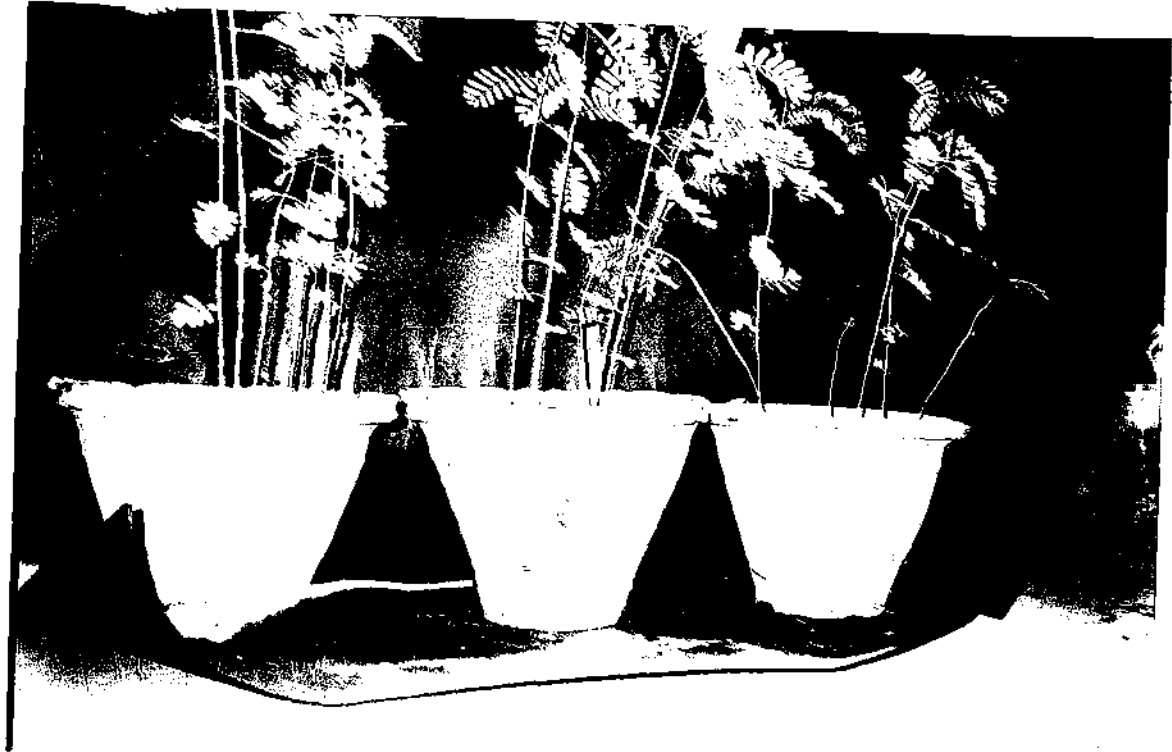


Plate 17



Plate 18

Table 32: Effect of inoculation of Azorhizobial isolates on plant height (cm) of *S. rostrata* under pot conditions

Treatments	Plant Height (cm)
R1	49.23
R2	37.66
R3	33.40
R4	31.06
S1	53.50
S2	71.73
ZB-SK-5	44.70
Control	32.83

CD (5%) - 13.71

SE - 04.52

Figure 36: Effect of inoculation of *Azorhizobium* isolates on plant height of *S.rostrata* under pot and field conditions

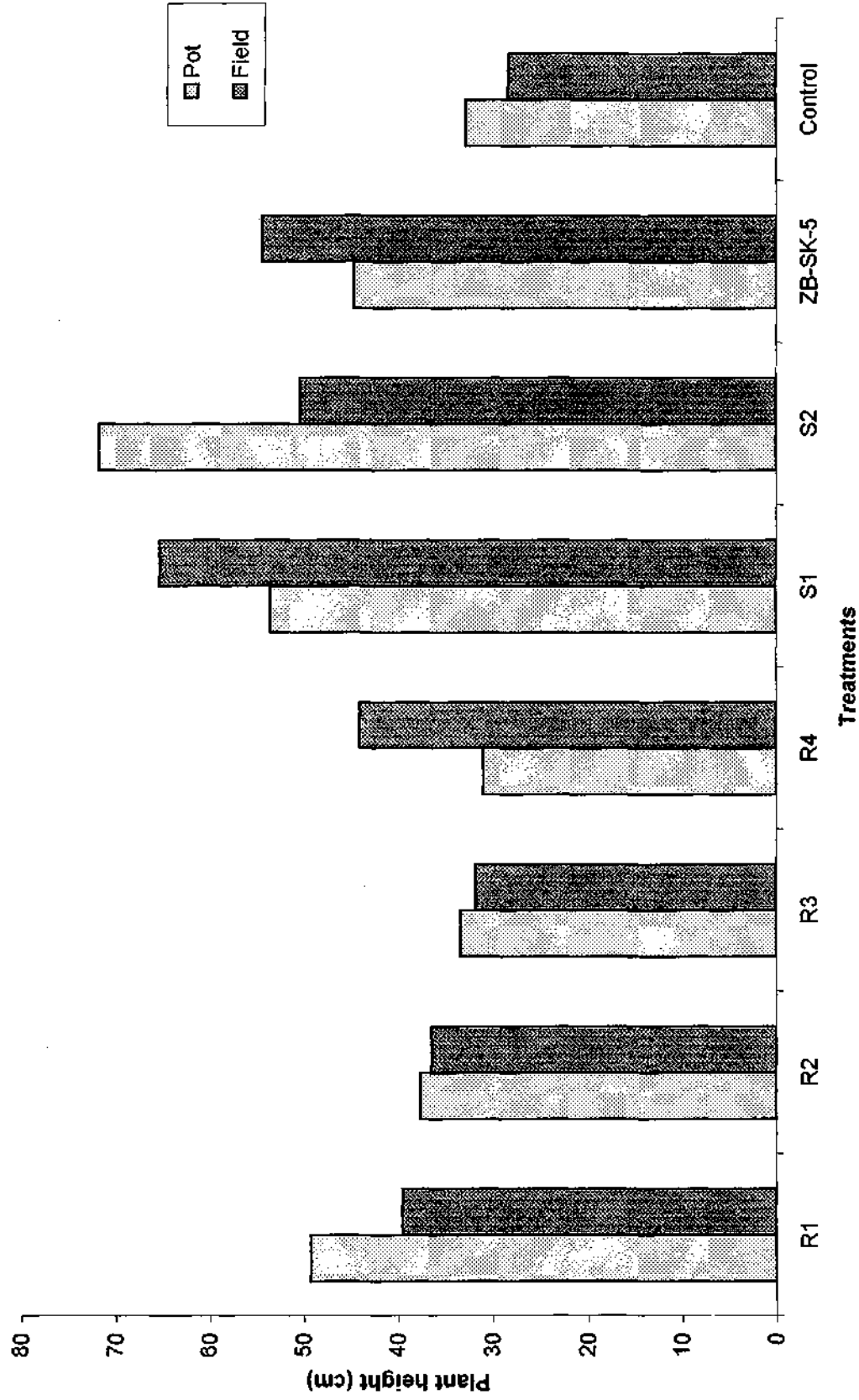
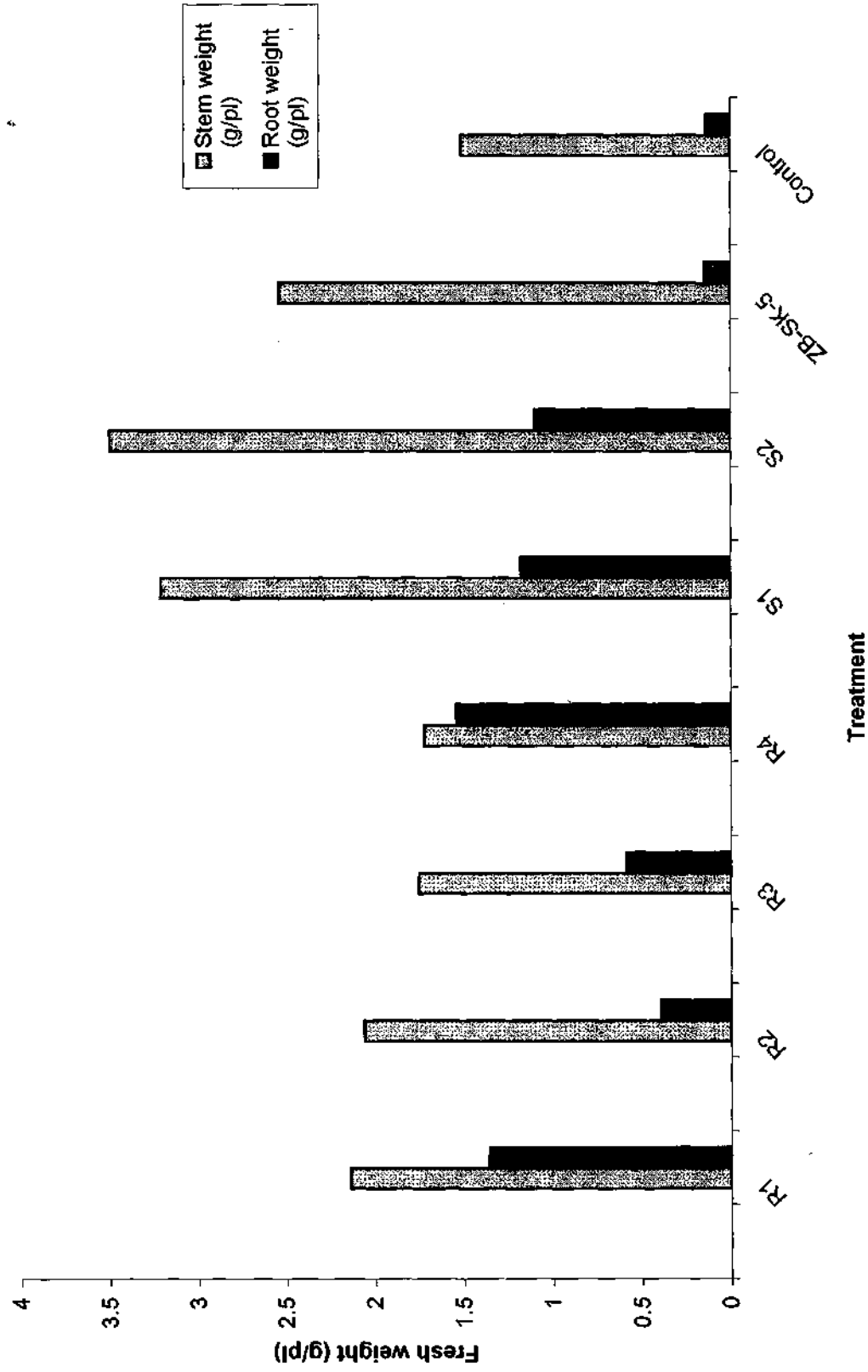


Table 33: Effect of inoculation of Azorhizobial isolates on stem and root fresh weight of *S.rostrata* under pot conditions

Treatment	Stem weight (g/pl)	Root weight (g/pl)
R1	2.14	1.36
R2	2.06	0.40
R3	1.75	0.59
R4	1.72	1.54
S1	3.21	1.18
S2	3.50	1.10
ZB-SK-5	2.54	0.143
Control	1.51	0.136
CD(5%)	0.67	0.4914
SE	0.22	0.3327

Figure 37: Effect of inoculation of *Azorhizobium* isolates on stem and root fresh biomass of *S. rostrata* under pot conditions



4.3.1.3 Root biomass

The root biomass ranged from 0.136-1.54 g (Table 33 and figure 37). All azorhizobial isolates showed a nonsignificant increase in root biomass over the control. The difference among these isolates was also observed to be nonsignificant. The maximum root biomass was recorded by R₄ followed by R₁. The root biomass was found to increase due to increased nitrogen available to the plants.

4.3.1.4 Stem nodules

The stem nodule number, fresh weight and dry weight were recorded (Table 34 and figures 39 and 40). The stem nodule number ranged from 0.33-8.0 nodules. The nodule number was found to be significantly better in all isolates over the control except R₁, R₂ and *A. caulinodans* ZB-SK-5 that were observed to be statistically at par with control (Plate 19). The best performance was given by R₃ amongst all isolates followed by S₁. Isolates R₃, R₄, S₁ and S₂ isolates were found to be significantly better over R₁ and the reference culture while the above isolates were statistically at par with each other.

The nodule fresh weight range was from 0.0045-0.074 g. All azorhizobial isolates performed significantly better over the uninoculated control. Maximum fresh weight was recorded by S₂ followed by R₃. Both stem and root isolates performed significantly better over the reference culture. Among the root isolates R₄ performed best while the rest of the isolates were statistically at par with each other. The difference between both stem isolates was also nonsignificant.

Table 34: Effect of inoculation of *Azorhizobium* isolates on stem nodules of *S. rostrata* under pot conditions

Treatments	Nodule no./pl	Nodule fresh weight(g/pl)	Nodule dry weight(g/pl)
R1	1.0	0.0152	0.009
R2	4.0	0.0308	0.0104
R3	8.0	0.0704	0.0252
R4	5.0	0.048	0.018
S1	6.3	0.0618	0.0259
S2	5.0	0.074	0.032
ZB-SK-5	1.0	0.0075	0.0015
Control	0.3	0.0045	0.0017

CD (5%) 3.89 0.044 0.018

SE 1.28 0.014 0.005

The stem nodule dry weight varied from 0.009-0.032 g. All the isolates produced significantly more dry weight over the control except R₁ that was statistically at par with the control. Among the root isolates R₃ produced maximum dry weight while rest of the root isolates were statistically at par with each other. S₂ produced maximum dry weight followed by S₁. All isolates showed significantly better results over the reference strain except R₁ that was at par with the reference strain. Kalidurai and Kannaiyan (1991) have reported that *S.rostrata* produces higher number and biomass of the stem nodules on 60th days after sowing which showed an increasing trend due to inoculation with *A.caulinodans* isolates.

4.3.1.5 Root nodules

The root nodule number, fresh and dry weight were studied (Table 35 and figures 41 and 42). The nodule number in isolate inoculated plants was found to be statistically at par with the control plants except for S₁ and S₂ that produced significantly higher nodule number over the uninoculated control (Plate 20). The nodule number ranged from 4.0-17.0 nodules plant⁻¹. The best performer amongst all was found to be S₁ followed by S₂. The reference strain showed at par results with all the root isolates while both stem isolates were observed to be significantly better over the *A.caulinodans* ZB-SK-5.

The root nodule fresh weight ranged from 0.0246-0.1758 g. All isolates gave a nonsignificant increase in fresh weight over the control except the stem isolates that that were significantly better over the control. The reference strain performed statistically at par to all the root isolates used while the stem isolates

Plate 19: Effect of inoculation of *A.caulinodans* isolates on stem nodulation status of *S.rostrata* grown under pot conditions

Plate 20: Effect of inoculation of *A.caulinodans* isolates on root nodulation status of *S.rostrata* grown under pot conditions

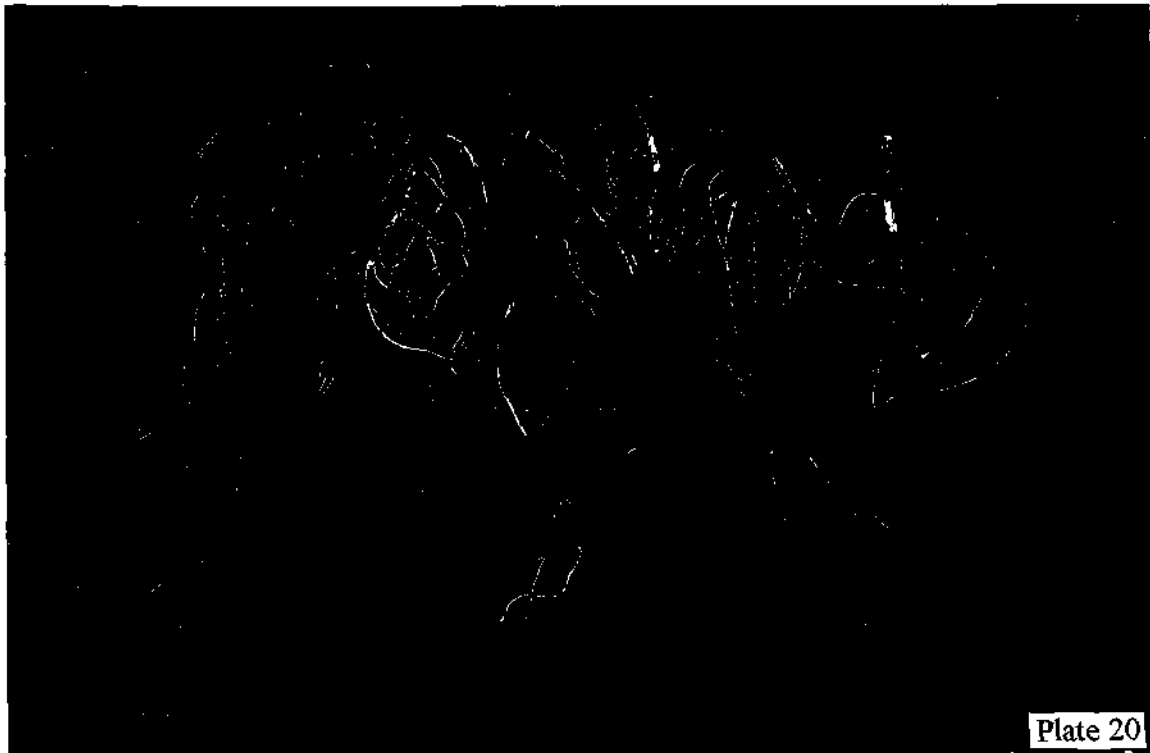
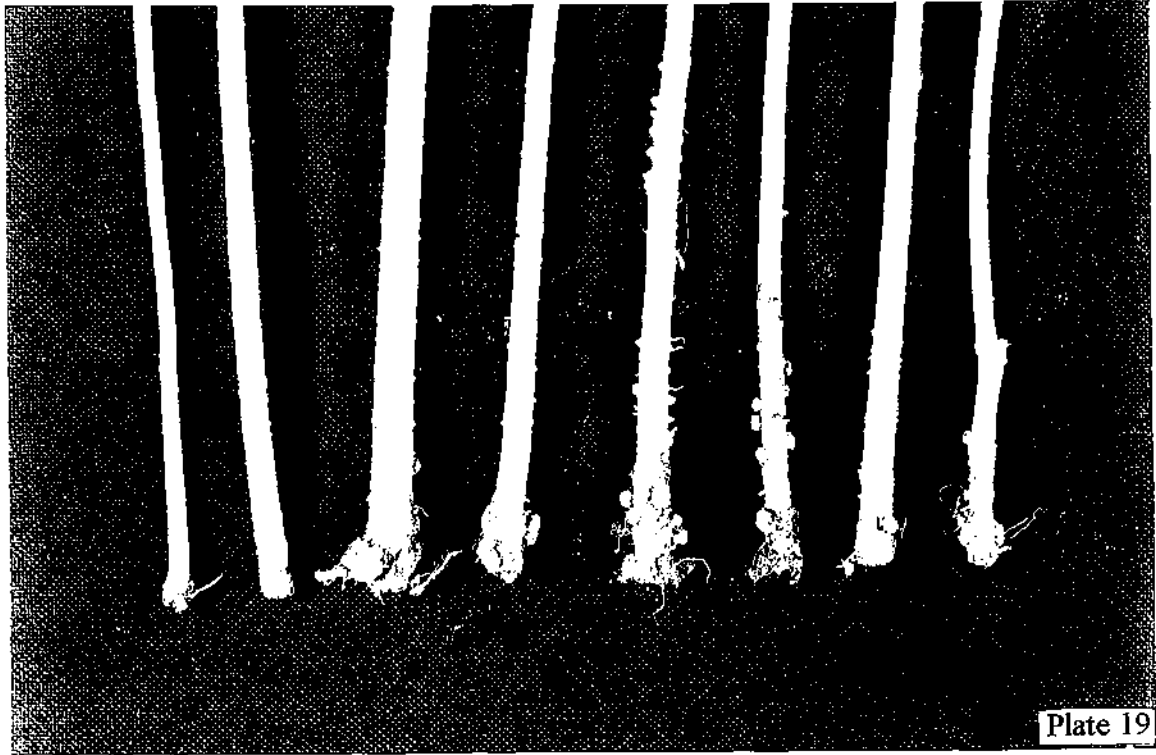


Table 35: Effect of inoculation of *Azorhizobium* isolates on root nodules of *S. rostrata* under pot conditions

Treatments	Nodule no./pl	Nodule fresh weight(g/pl)	Nodule dry weight(g/pl)
R1	10.3	0.0711	0.0257
R2	8.3	0.0626	0.0213
R3	10.7	0.0886	0.025
R4	10.3	0.087	0.019
S1	17.0	0.1625	0.0325
S2	16.0	0.1758	0.0347
ZB-SK-5	7.0	0.0854	0.013
Control	4.0	0.0246	0.0025

CD (5%) 7.49 0.075 0.017

SE 2.47 0.024 0.005

were observed to be significantly better over the reference strain. S₂ was recorded as the best performer followed by S₂. Both the stem isolates were observed to be statistically at par with each other.

The nodule dry weight varied from 0.0025-0.0347 g. All the isolates produced significantly more dry weight over the uninoculated control. Maximum dry weight was recorded by S₂ followed by S₁. The root isolates showed no significant difference between each other, similarly the stem isolates were also found to be statistically at par with each other. The stem isolates performed significantly better over the root isolates. All isolates performed significantly better over the reference culture except R₄ that was observed to be statistically at par with the reference strain. The increase in number of root nodules of *S.rostrata* was observed which may be attributed to the seed inoculation of Azorhizobial isolates that increases the chances of azorhizobia to infect the roots and form nodules.

4.3.1.6 Root nitrogenase activity

The root nodule nitrogenase activity as measured by the acetylene reduction assay, ranged from 52.47-503.55 nM C₂ H₂ plant⁻¹ h⁻¹ (Table 36 and figure 43). All isolates except R₁ and the reference strain (which were statistically at par with the control) produced significantly better results over the control. All root isolates were found to be statistically at par with each other except R₁ that was significantly lesser than R₃ and R₄. Both stem isolates were also found to be statistically at par with each other but were significantly better over the root isolates as well as the reference strain. The root nodules of *S.rostrata* produces

Table 36: Effect of inoculation of *Azorhizobium* isolates on root nodules nitrogenase activity (nM/plant/hour) of *S.rostrata* under pot conditons

Treatments	Mean
R1	96.07
R2	227.36
R3	227.37
R4	192.39
S1	466.39
S2	503.55
ZB-SK-5	134.08
Control	52.47

CD (5%) 117.119

SE 38.61

Table 37: Percent establishment of inoculated *Azorhizobium* isolates on *S.rostrata* under pot conditions

Treatments	Establishment
R1	33.3
R2	48.1
R3	62.9
R4	66.6
S1	85.1
S2	81.4
ZB-SK-5	51.0
Control	0.0

CD (5%) 10.39

SE 3.420

highest nitrogenase activity on 40th day (Kalidurai and Kannaiyan, 1991) and inoculation with the *A.caulinodans* isolates increases the nitrogenase activity due to formation of increased number of the functional nodules.

4.3.1.7 Competitiveness of inoculated isolates

All isolates were found to be established in the rhizosphere soil and were observed to be more competitive over the uninoculated control (Table 37 and figure 44). The competitiveness ranged from 33.3-85.1%. S₁ was observed to be most established amongst all isolates while R₁ was the least established isolate. Both stem isolates performed significantly better over all the root isolates. Among the root isolates R₄ was the most established. The stem isolates showed statistically at par results regarding competitiveness. The reference strain showed 51% establishment under pot conditions. The local isolates performed better over the known Azorhizobial isolates in literature, which indicates that the local strains are more adapted to the local soil and climatic conditions and thus outdo the known isolates.

4.3.1(b) Field Experiment

4.3.1.8 Plant Height

The height of plants varied from 28.35-65.38 cm (Table 38 and figure 36). R₄, S₁, S₂ and the reference culture were found to be significantly better over the control. All root isolates were found to be statistically at par with each other while among stem isolates S₁ performed significantly better over S₂ as well as over root isolates. The reference strain was found to be significantly better over all root isolates and the stem isolates were statistically at par with *A.caulinodans*

Table 38: Effect of inoculated Azorhizobial isolates on plant height of *S.rostrata* under field conditions

Treatments	Plant height (cm)
R1	39.53
R2	36.52
R3	31.81
R4	44.07
S1	65.38
S2	50.37
ZB-SK-5	54.38
Control	28.35

CD (5%) 12.32

SE 4.52

ZB-SK-5. Isolate S₁ was the best performer amongst all isolates followed by the reference strain. The increased plant height upon *A. caulinodans* isolates inoculation was due to the increased amount of nitrogen available to the plant via nitrogen fixation by the stem and root nodules of *S. rostrata*

4.3.1.9 Stem fresh Biomass

Table 39 and figure 38 showed the stem fresh weight that ranged from 5.0-21.54 g. All isolates produced significantly more amount of fresh biomass over the control except R₂ and the reference culture. Among the root isolates R₃ worked best and was significantly better over R₂ and R₄ while was statistically at par with R₁. S₁ was recorded as the best performer followed by R₃. S₁ was also found to be significantly better over S₂. Kannaiyan and Kalidurai (1989) have reported that *S. rostrata* produces significant amount of biomass that increases due to *Azorhizobium caulinodans* inoculation.

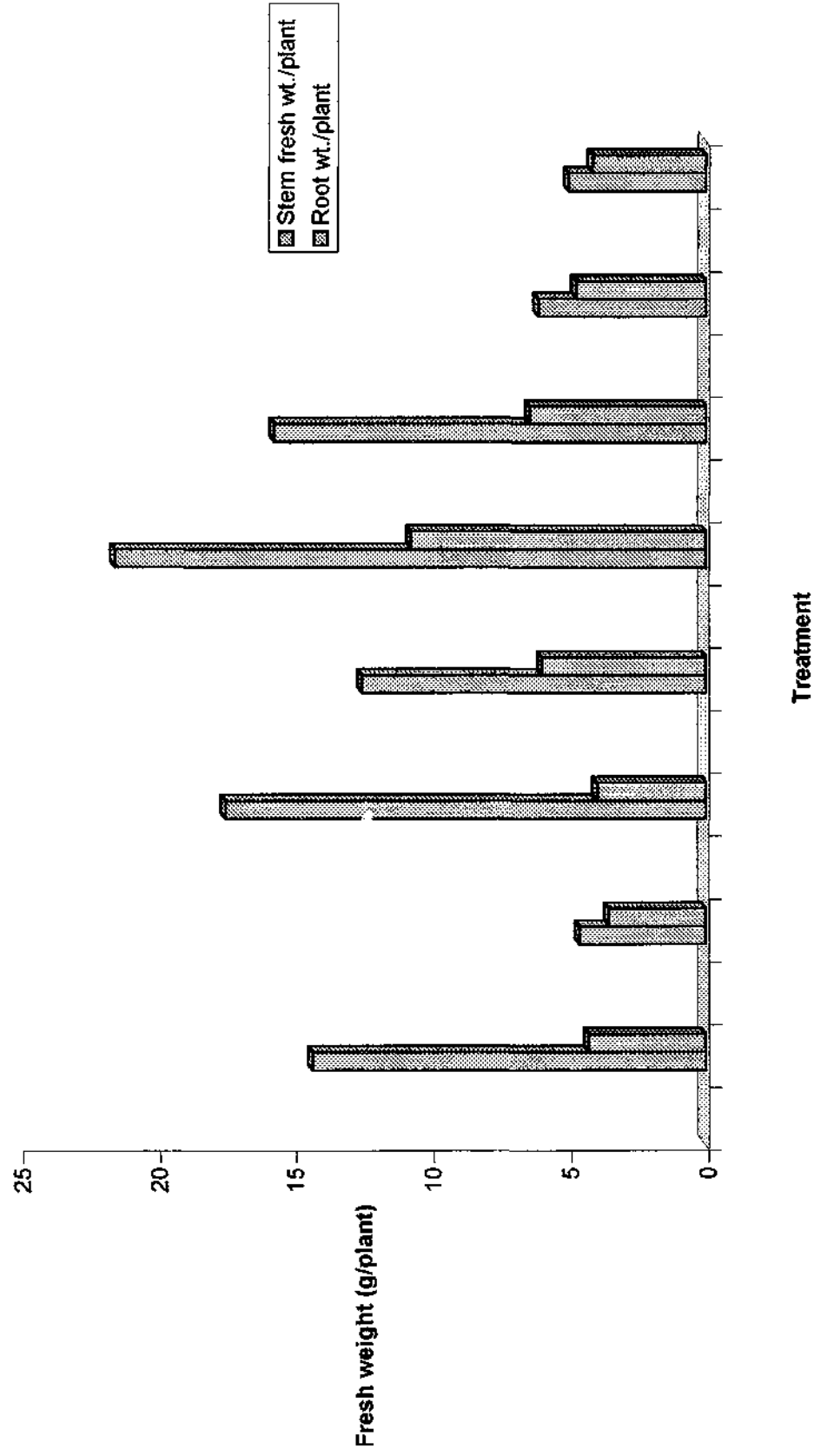
4.3.1.10 Root Biomass

The root biomass varied from 3.52-10.69 g (Table 39 and figure 38). All isolates produced statistically at par results with the control except S₁. Thus a nonsignificant increase in root biomass was observed in all azorhizobial isolates over the control. All root isolates were statistically at par with each other. S₁ performed significantly better over S₂ and was the best performer amongst all isolates. The reference culture gave statistically at par values with the root isolates.

Table 39: Effect of inoculation of *Azorhizobium* isolates on stem and root fresh weight of *Sesbania rostrata* under field conditions

Treatments	Stem fresh wt./plant	Root wt./plant
R1	14.30	4.273
R2	4.61	3.523
R3	17.50	3.966
R4	12.50	5.970
S1	21.54	10.690
S2	15.71	6.416
ZB-SK-5	6.10	4.733
Control	5.00	4.146
CD (5%)	3.44	3.872
SE	1.134	1.270

Figure 38: Effect of inoculation of *Azorhizobium* isolates on stem and root weight of *S.rostrata* under field conditions



4.3.1.11 Stem nodules

The nodule number, fresh and dry weight of stem nodules was recorded (Table 40 and figures 39 and 40) that revealed that the nodule number ranged from 9.55-75.10 per plant. Isolate R₄, S₁ and S₂ gave significantly better nodule number over the uninoculated control (Plate 21). Rest of the isolates showed a nonsignificant increase in nodule number over the control. All root isolates were found to be statistically at par with each other as well as with the reference strain. S₁ was the best performer and was significantly better over S₂.

The nodule fresh weight ranged from 0.13-1.72 g. All isolates produced significantly more amount of fresh weight over the control. The reference culture was observed to be statistically at par with the root isolates and S₂. The difference between the root isolates was nonsignificant and similar situation resides among the stem isolates. Maximum fresh weight was produced by S₁ followed by S₂.

The range of nodule dry weight was from 0.05-0.63 g. All isolates performed significantly better over the control. The reference culture produced statistically at par results with all root isolates except R₃. The results among root isolates were also statistically at par with each other. S₁ produced maximum amount of fresh weight followed by S₂. The stem nodule number increases due to seed inoculation by the *Azorhizobium* isolates as the azorhizobia present on the surface could cause nodulation at the infection foci of the germinating *Sesbania* seedlings. The increase in number also increases the fresh and dry weight of the stem nodules.

Table 40: Effect of inoculation of *Azorhizobium* isolates on stem nodules of *S. rostrata* under field conditions

Treatments	Nodule no./pl	Nodule fresh weight(g/pl)	Nodule dry weight(g/pl)
R1	12.66	0.97	0.39
R2	21.37	0.95	0.15
R3	31.10	1.03	0.21
R4	23.77	1.27	0.20
S1	75.10	1.72	0.63
S2	47.77	1.47	0.54
ZB-SK-5	23.88	0.99	0.13
Control	9.55	0.13	0.01

CD (5%) 20.27 0.57 0.089

SE 6.68 0.189 0.029

Figure 39: Effect of inoculation of *Azorhizobium* isolates on stem nodule number of *S.rostrata* under pot and field conditions

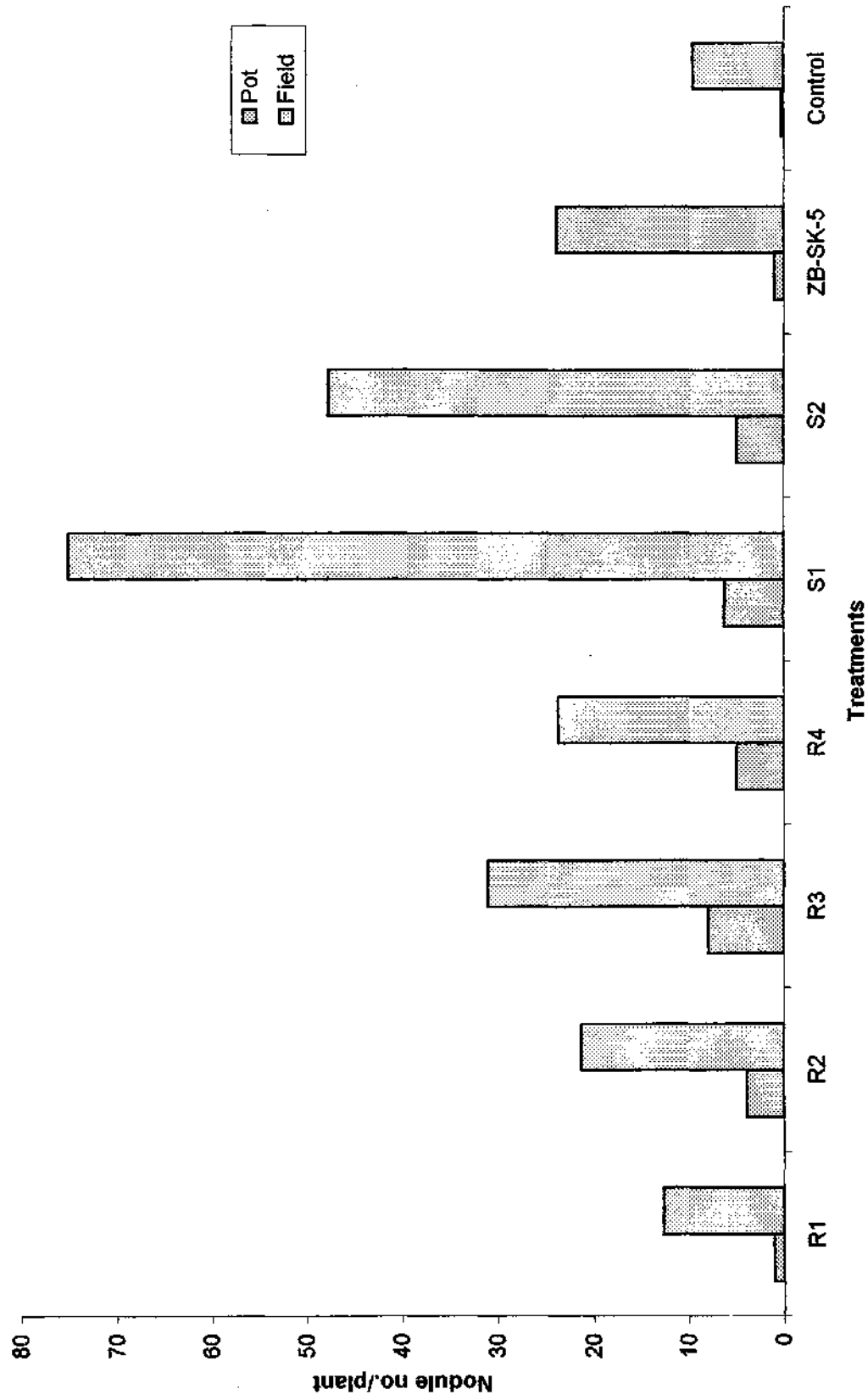
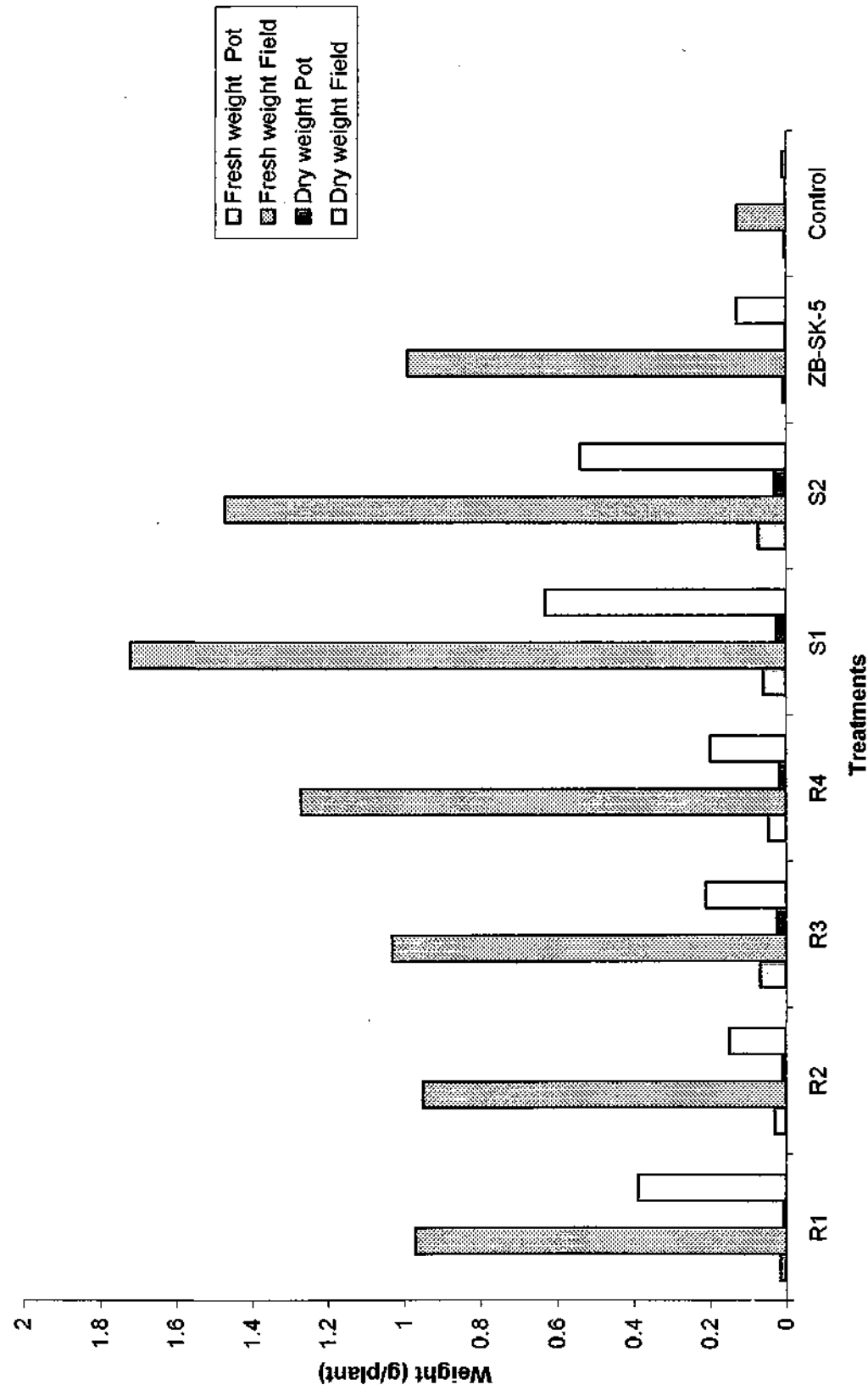


Figure 40: Effect of inoculation of *Azorhizobium* isolates on stem nodule fresh and dry weight of *S.rostrata* under pot and field conditions



4.3.1.12 Root nodules

Table 41 and figures 41 and 42 revealed the effect of azorhizobial isolates on root nodule number, fresh and dry weight in *S.rostrata* (Plate 22). The number of root nodules observed ranged from 20.3-63.4 per plant. Isolate R₄, S₁ and S₂ performed significantly better over the control. The root isolates were found to be statistically at par with each other and with the control except R₄ that was significantly better over other root isolates. Both stem isolates were also found to be statistically at par with each other. Maximum number of root nodules was recorded by S₁ followed by S₂.

The nodule fresh weight was found to vary from 0.86-1.76 g. All isolates except R₁, and R₃ performed significantly better over the control. The difference between the root isolates was nonsignificant and was significantly lower than the stem isolates except R₄ that was statistically at par. Both stem isolates had statistically at par values. S₂ was recorded as the best performer followed by S₁.

The nodule dry weight ranged from 0.15-0.55 g. Both stem isolates and R₄ performed significantly better over the control. Among root isolates R₄ performed best and was statistically at par with R₃. Among stem isolates S₂ performed significantly better over S₁ as well as over the reference strain. The best performance was given by S₂ followed by S₁ and *A.caulinodans* ZB-SK-5. The root nodule number increased due to infection by the azorhizobia present on the surface of the seeds which culminated to increase in fresh and dry weight of the root nodules per plant.

Table 41: Effect of inoculation of *Azorhizobium* isolates on root nodules of *S.rostrata* under field conditions

Treatments	Nodule no./pl	Nodule fresh weight(g/pl)	Nodule dry weight(g/pl)
R1	28.0	1.01	0.15
R2	24.1	1.27	0.22
R3	21.2	0.97	0.26
R4	40.3	1.37	0.34
S1	59.5	1.59	0.43
S2	63.4	1.76	0.55
ZB-SK-5	28.3	1.35	0.43
Control	20.3	0.86	0.22

CD (5%) 9.75 0.27 0.11

SE 3.21 0.09 0.037

Figure 41: Effect of inoculation of *Azorhizobium* isolates on root nodule number of *S.rostrata* under pot and field conditions

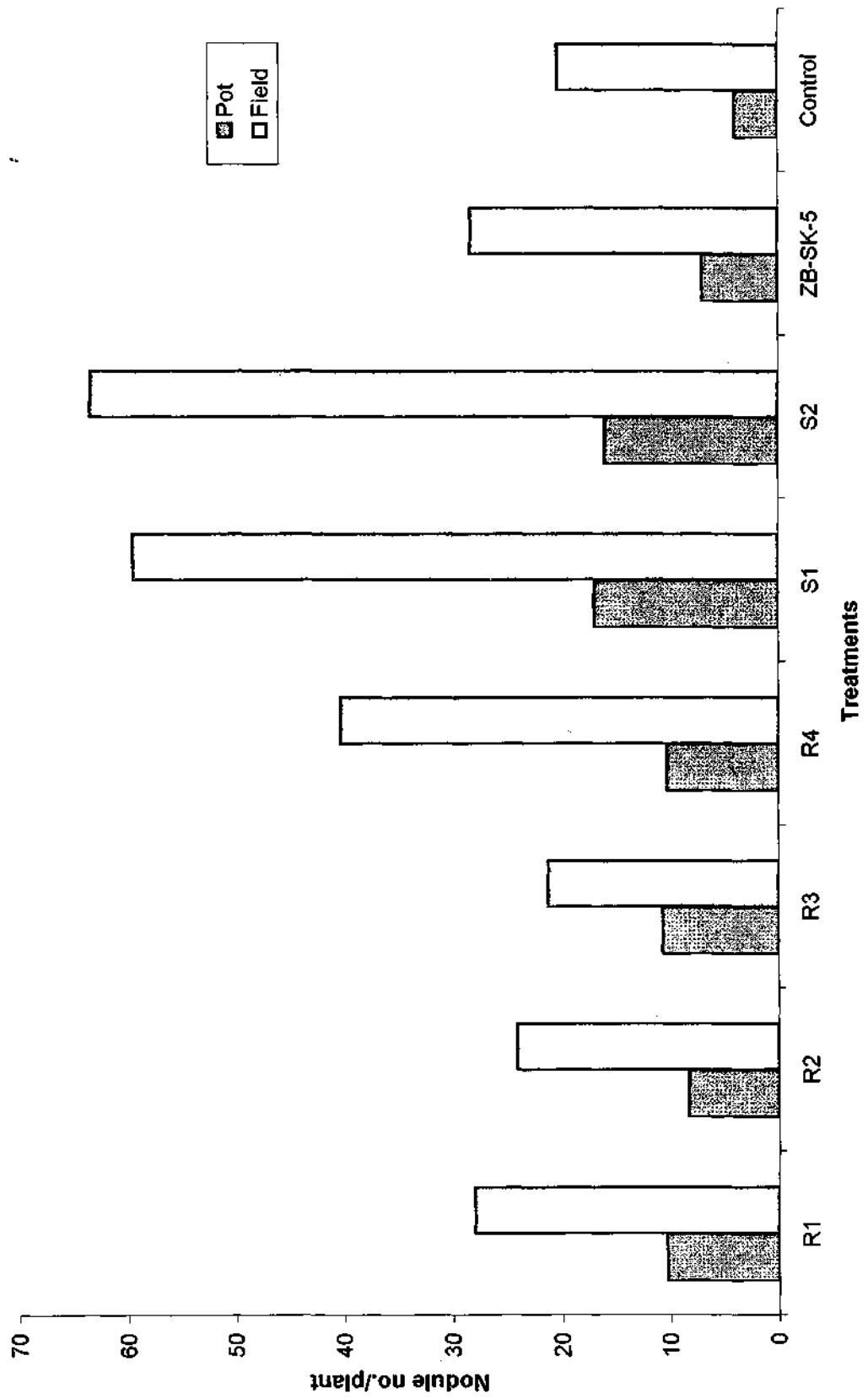


Figure 42: Effect of inoculation of *Azorhizobium* isolates on root nodule fresh and dry weight of *S.rostrata* under pot and field conditions

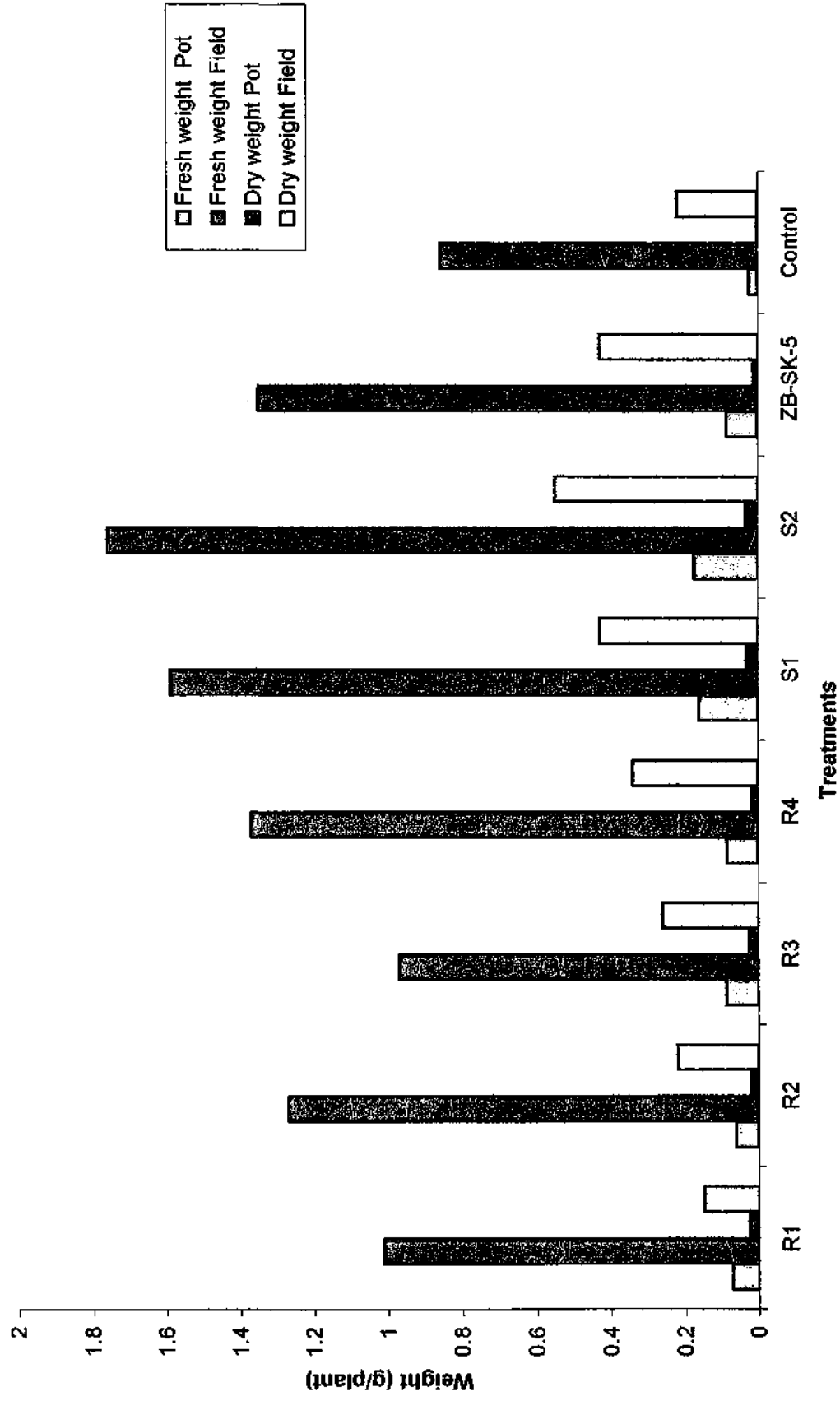


Plate 21: Effect of inoculation of *A.caulinodans* isolates on stem nodulation status of *S.rostrata* grown under field conditions

Plate 22: Effect of inoculation of *A.caulinodans* isolates on root nodulation status of *S.rostrata* grown under field conditions



Plate 21



Plate 22

4.3.1.13 Root nitrogenase activity

The root nitrogenase activity was observed to range from 343.97-2046.34 nM C₂ H₂ plant⁻¹ h⁻¹ (Table 42 and figure 43). Most of the azorhizobial isolates produced significantly better results over the uninoculated control except R₂, R₃ and *A.caulinodans* ZB-SK-5. The maximum nitrogenase activity was recorded by S₁ followed by S₂. Among the root isolates R₄ performed best. The difference between stem isolates was nonsignificant but they showed significantly better values over the root isolates and the reference culture. The root nodules of *S.rostrata* produced maximum root nitrogenase activity on the 40th DAS and *Azorhizobium* isolates inoculation increases the number of root nodules as well as the functional nodules inhabited by the bacteria also increases that gave increase in amount of acetylene reduced by the treated plants.

4.3.1.14 Competitiveness of inoculated isolates

By using the antibiotic resistance markers it was found that all the isolates studied competed with the indigenous rhizobacteria to establish themselves in the rhizosphere soil and form nodules (Table 43 and figure 44). However the extent of establishment varied from 30.3-77.7%. S₁ has been found to have maximum establishment value of 77.7% followed by S₂ whereas R₁ was found to have least establishment indicating that both stem isolates and R₄ have the maximum competitiveness as compared to the natural flora. The reference strain was found to be statistically at par with the R₁ and R₂ isolates indicating approximately 50% establishment in *S.rostrata* nodules. Thus the local isolates should be used, as they possess better nitrogen fixing capacities and far better

Table 42: Effect of inoculation of Azorhizobial isolates on root nitrogenase activity of *Sesbania rostrata* under field conditions

Treatments	Root Nitrogenase activity (nM/plant/hr)
R1	743.33
R2	402.27
R3	437.45
R4	1119.36
S1	2046.34
S2	2011.36
ZB-SK-5	542.19
Control	343.97

CD (5%) 213.166

SE 70.27

Figure 43: Effect of inoculation of *Azorhizobium* isolates on root nitrogenase activity of *S. rostrata* under pot and field conditions

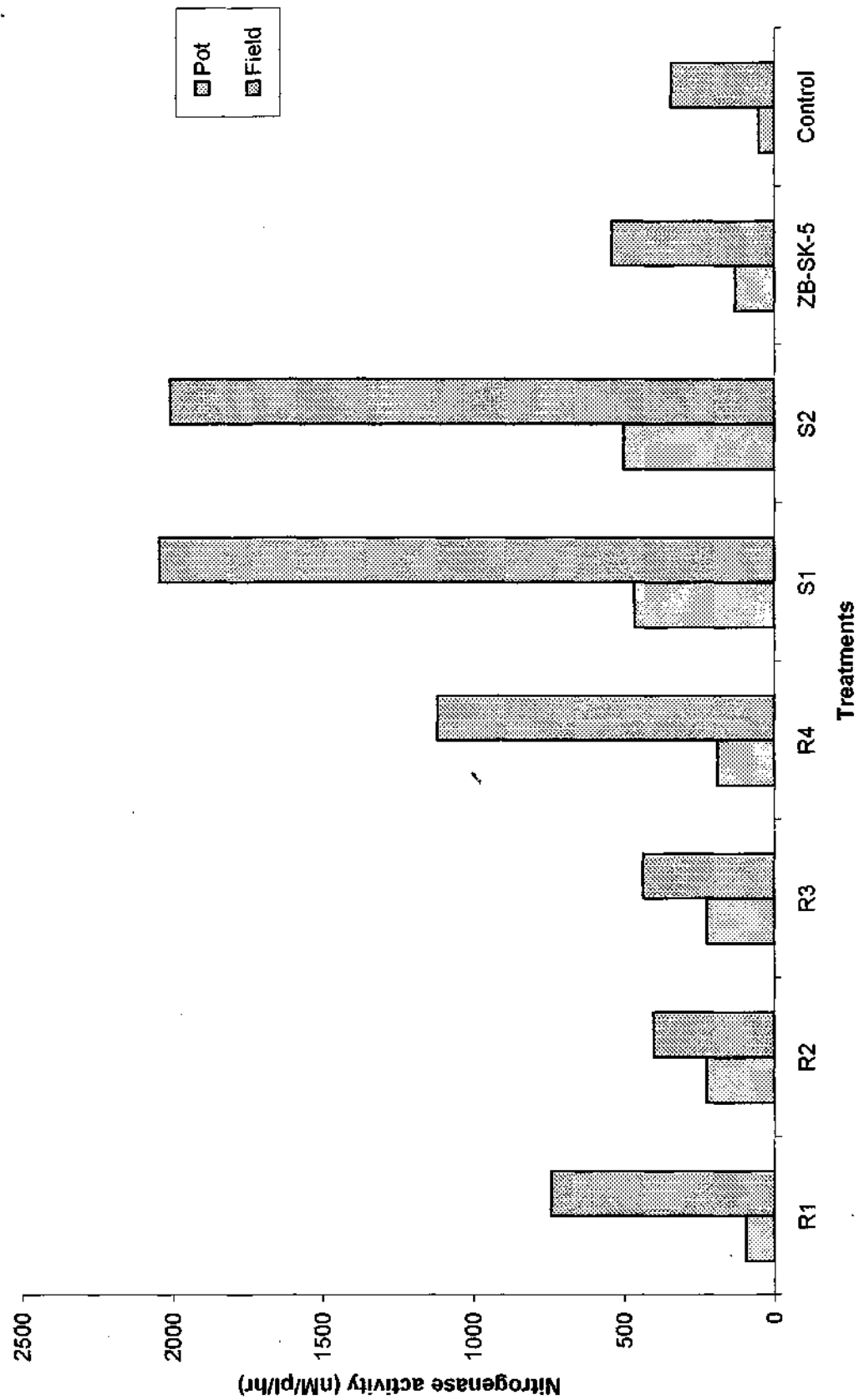


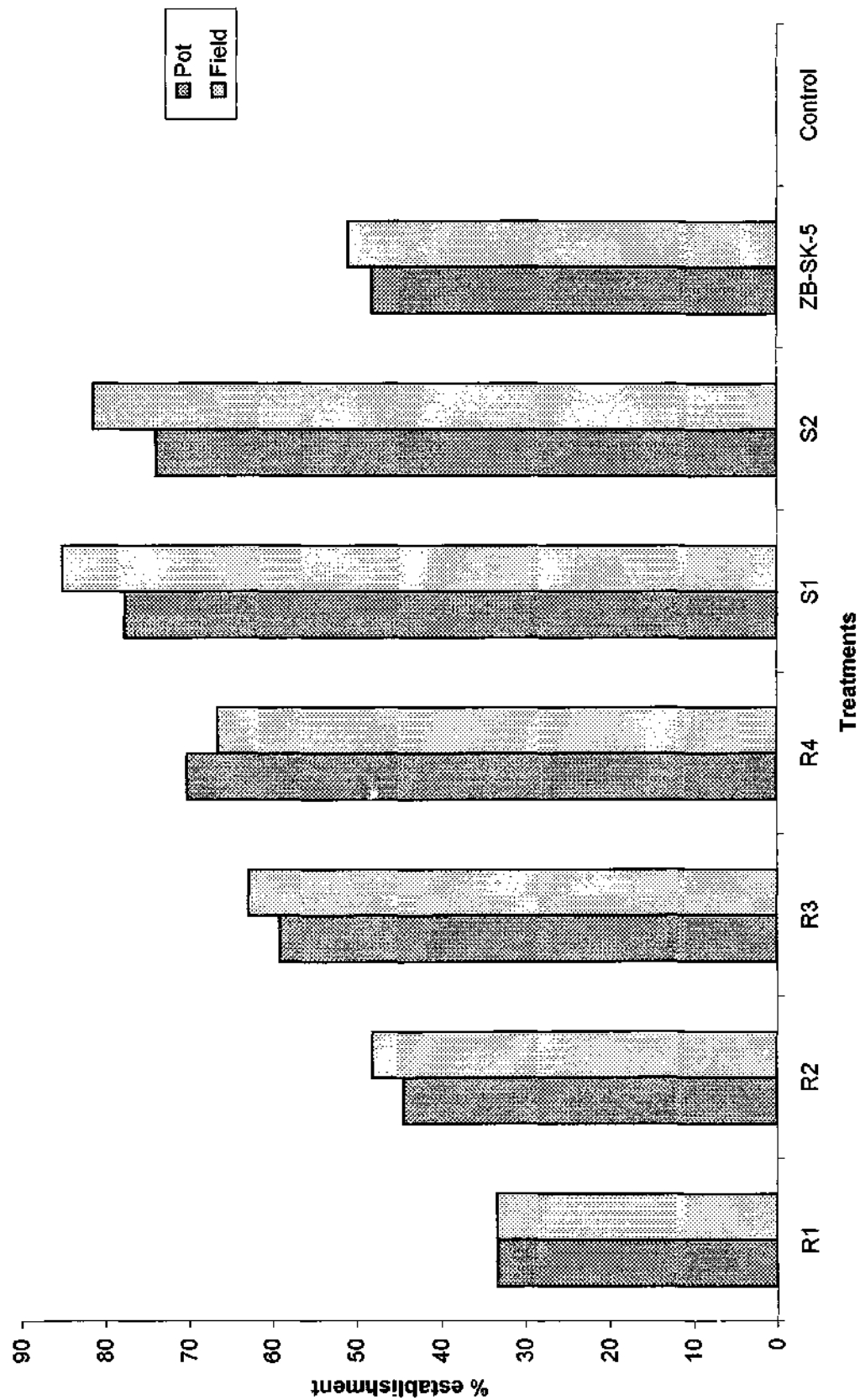
Table 43: Percent establishment of inoculation of Azorhizobial isolates on *Sesbania rostrata* under field conditions

Treatments	% establishment
R1	33.30
R2	44.40
R3	59.20
R4	70.30
S1	77.70
S2	73.99
ZB-SK-5	48.10
Control	0.00

CD (5%) 10.497

SE 3.46

Figure 44: Percent establishment of inoculated *Azorhizobium* isolates in *S.rostrata* under pot and field conditions



establishment percentage. The local isolates were well adapted to the surrounding environmental conditions and thus have increased survival rate over the reference strain and were able to compete well with the native microflora.

4.3.1.15 Yield

The yield obtained at maturation of pods varied from 14.16-47.6 g (Table 44 and figure 45). The *Azorhizobium* inoculated plots gave significantly more grain yield than the uninoculated control plots except R₂, R₃ and R₄ that showed a nonsignificant increase in yield over the control. S₁ produced maximum grain yield followed by S₂. Among the root isolates R₁ produced statistically better results over R₂ while was at par with rest of the root isolates. The stem isolates performed significantly better over all root isolates. All root isolates and the stem isolate S₂ were found to be statistically at par with the reference strain. Among the stem isolates S₁ performed significantly better over S₂. The increased ability of expression of the nitrogenase enzymes and thus increased nitrogen fixation and potential to compete well in the rhizosphere soil of the *Azorhizobial* isolates over the reference strain led towards increase in biomass and grain yield.

4.4 Effect of inoculation of *Azorhizobium caulinodans* isolates along with the reference culture on rice cv PR-115 under field conditions

The four isolates as screened by the pot and field experiments were used to study effect on rice PR-115 under the field conditions. Two root (R₃ and R₄) and two stem (S₁ and S₂) isolates as well as the reference culture *Azorhizobium caulinodans* ZB-SK-5 were seed inoculated and effects were studied at two nitrogen levels of N full recommended dose and half N dose.

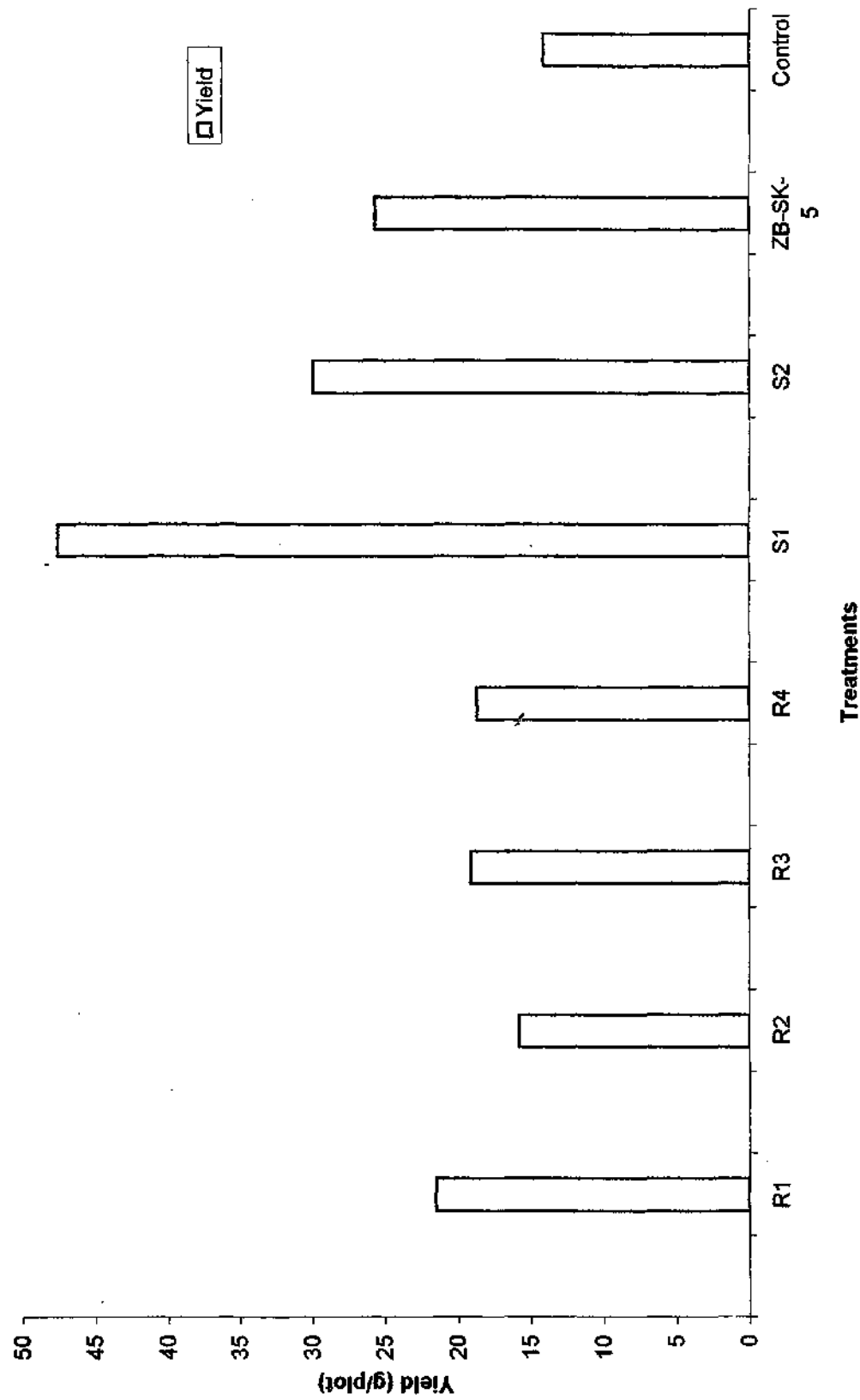
Table 44: Effect of inoculation of Azorhizobial isolates on yield of *Sesbania rostrata* (g/plot) under field conditions

Treatments	Yield
R1	21.49
R2	15.80
R3	19.11
R4	18.71
S1	47.60
S2	29.97
ZB-SK-5	25.66
Control	14.16

CD (5%) 5.819

SE 1.918

Figure 45: Effect of inoculation of *Azorhizobium* isolates on yield of *S.rostrata* under field conditions



4.4.1 The plots in which *S.rostrata* seeds inoculated with the *Azorhizobium caulinodans* isolates were grown for 45 days; the fresh biomass, plant height, root and stem nodule number, fresh and dry weight and nitrogenase activity was studied.

4.4.1.1 Fresh Biomass

The *S.rostrata* plant fresh biomass ranged from 1.64-2.63 Kg (Table 45). All the azorhizobial isolates as well as the reference culture produced significantly more amount of fresh biomass over the uninoculated control. The difference between the all isolates was observed to be nonsignificant. The S₁ isolate produced the maximum fresh biomass followed by S₂. All isolates performed at par statistically with the reference culture.

4.4.1.2 Plant height

The plant height of the *S.rostrata* plants was observed to vary from 103-154 cm (Table 45). All *Azorhizobium caulinodans* isolates performed significantly better over the control except R₄ that was statistically at par with the control. Maximum plant height was recorded by S₂ followed by S₁. The reference culture was statistically at par with all isolates except R₄ that was significantly lower than the reference strain.

4.4.1.3 Stem nodules

Table 46 revealed the stem nodule number, fresh and dry weight .The nodule number ranged from 34.6-295 nodules per plant. Both stem isolates performed significantly better over the control while rest of the isolates and the reference strain recorded a nonsignificant increase in nodule number over the

Table 45: Effect of Azorhizobial isolates inoculation on biomass (Kg/m²) and plant height (cm) of *Sesbania rostrata* under field conditions

Treatments	Fresh Biomass	Plant height (cm)
R3	2.43	139
R4	2.45	106
S1	2.63	141
S2	2.60	154
ZB-SK-5	2.16	139
Control	1.64	103

Table 46: Effect of inoculation of Azorhizobial isolates on stem nodules of *Sesbania rostrata* under field conditions

Treatments	Nodule no./pl	Nodule fresh wt.(g/pl)	Nodule dry wt.(g/pl)
R3	88.00	0.4	0.2
R4	57.60	0.53	0.26
S1	282.60	1.16	0.45
S2	295.00	1.92	0.58
ZB-SK-5	80.60	0.51	0.26
Control	34.60	0.34	0.14
CD (5%)	56.830	0.23	0.11
SE	18.040	0.07	0.03

control. The stem isolates were statistically at par with each other. The stem isolates were found to be significantly better over the root isolates. S_2 was recorded to have the maximum number of nodules followed by S_1 .

The stem nodule fresh weight varied from 0.34-1.92 g. Both stem isolates performed significantly better over the control while R_4 and *A. caulinodans* ZB-SK-5 were observed to be statistically at par with the control. Both root isolates were observed to be statistically at par with each other while among stem isolates S_2 performed significantly better over S_1 . The stem isolates were also significantly better over root isolates and over the reference strain.

The nodule dry weight ranged from 0.14-0.58 g. All azorhizobial isolates except R_3 performed significantly better over the control. Both root isolates were observed to be statistically at par with each other. Among stem isolates S_2 performed significantly better over S_1 . Stem isolates performed significantly better over the root isolates and over the reference strain. The best performer amongst all was recorded as S_2 followed by S_1 . The Shoot nodule number increases that result in increase in fresh and dry weight of stem nodules per plant. The increase in nodule number could be due to increased infection of the emerging seedling shoot by the inoculated azorhizobia.

4.4.1.4 Shoot nitrogenase activity

The shoot nitrogenase activity as observed by ARA ranged from 699.60-3498.02 $\text{nMC}_2\text{H}_2\text{ plant}^{-1}\text{ h}^{-1}$ (Table 48). All isolates showed a nonsignificant increase in the nitrogenase activity over the uninoculated control. The results among isolates were also observed to have no significant difference but the stem

isolates showed a sharp yet nonsignificant increase over the root isolates. Maximum shoot nitrogenase activity was recorded by S₁ followed by S₂. The shoot nodules gave higher nitrogenase activity over the root nodules because the stem nodules are away from competition and are presumed to be photosynthetic (Fleischmann, 1991). Kalidurai and Kannaiyan (1991) have reported that the shoot nodules show maximum nitrogenase activity 60th DAS.

4.4.1.5 Root nodules

Table 47 revealed the root nodule number, fresh and dry weight. The root nodule number varied from 33.4-125.5. All the isolates performed significantly better over the control. The maximum number of root nodules was observed in S₂ followed by S₁. No significant difference was observed among the root isolates while among the stem isolates S₂ performed significantly better over S₁. The reference strain showed at par statistic values with the root and stem isolates except S₂ that was significantly better over reference strain.

The nodule fresh weight ranged from 0.22-1.02 g. All azorhizobial isolates as well as reference strain were observed to be significantly better over the uninoculated control. Both root isolates were found to be statistically at par with each other and with the reference culture. Among stem isolates S₂ produced significantly high nodule fresh weight over S₁ and was also best performer amongst all isolates.

The nodule dry weight was observed to vary from 0.11-0.45 g. All azorhizobial isolates produced significantly higher dry weight than the uninoculated control except R₃ that was statistically at par with reference strain.

Table 47: Effect of inoculation of *Azorhizobium* isolates on root nodules of *S.rostrata* under field conditions

Treatments	Nodule no./pl	Nodule fresh weight(g/pl)	Nodule dry weight(g/pl)
R3	72.9	0.48	0.14
R4	59.9	0.51	0.24
S1	74.5	0.56	0.29
S2	125.5	1.02	0.45
ZB-SK-5	64.7	0.51	0.24
Control	33.4	0.22	0.11

CD (5%) 18.74 0.18 0.15

S.E. 5.95 0.05 0.04

Amongst stem isolates S_2 produced significantly high nodule dry weight over S_1 and was also the best performer amongst all isolates. The root nodule number increase could have resulted from infection by the azorhizobia applied on the surface of the seeds during seed inoculation procedure that caused the increased fresh and dry weight measured per plant.

4.4.1.6 Root nitrogenase activity

The root nitrogenase activity ranged from 34.87-1603.26 nM C_2H_2 plant⁻¹ h⁻¹ (Table 48). All isolates produced significantly higher amount of nitrogenase activity over the control. R_4 performed significantly better over R_3 among root isolates while S_1 performed significantly better over S_2 amongst stem isolates. All isolates were observed to be significantly better over reference strain except R_3 , which was found to be significantly lower than the reference strain. The root nitrogenase activity increased on account of increase in number of nodules formed per plant as well as due to increase in number of functional nodules inhabited by azorhizobia.

4.4.1.7 Competitiveness of inoculated strain isolates

The extent of establishment of inoculated isolates (Table 49) ranged from 59.2-81.4%. The maximum establishment values were given by S_1 followed by S_2 . While the least establishment values were given by the reference strain. Both stem isolates and the R_4 isolates possessed maximum competitiveness as compared to the natural flora as well as the reference strain. The competitiveness was discussed on the basis of the antibiotic resistance markers screened for all isolates and the reference strain before the experiment.

Table 49: Percent establishment of Azorhizobial isolates in *S.rostrata* under field conditions

Treatments	% establishment
R3	62.9
R4	70.3
S1	81.4
S2	77.7
ZB-SK-5	59.2
Control	0.0

CD (5%) 12.5

SE 3.9

4.4.2 Effect of burial of *S.rostrata* and inoculation of *Azorhizobium caulinodans* isolates on rice cv PR-115 under field conditions

The 30 days old rice nursery was transplanted after inoculation with *Azorhizobium caulinodans* isolates on the field plots having buried *Sesbania* plants and the rest of the control where *S.rostrata* was not planted (Plate 23). Before harvesting data was recorded for several parameters such as plant height, tiller number, panicle length, dry weight of plant, grain weight, root dry weight, paranodule number, nitrogenase activity, establishment of isolates and the total yield.

4.4.2.1 Plant Height

Table 50 and figure 46 depicted that the average plant height that ranged from 84.66-93.16 cm. All azorhizobial isolates performed significantly better over the control except the reference culture and R₄ in half N and reference strain in full N plots that performed statistically at par with the control plants. Among root isolates R₃ showed significantly better results over R₄ while among stem isolates S₁ performed significantly better over S₂. The reference strain was found to be statistically at par with R₄ while the rest of the isolates performed significantly better over the reference strain. The better performer amongst all was the S₁ isolate followed by S₂. The isolates performed significantly better at full N plots over the half N plots.

4.4.2.2 Tiller number

The average tiller number varied from 9.66-12.66 (Table 51 and figure 47). The half N applied plants produced significantly more number of tillers over

Table 50: Effect of inoculation of azorhizobial isolates on plant height (cm) of rice cv PR-115 under field conditions

Treatment	Plant height		Mean
	1/2 N	N	
R3	90.0	93.0	91.50
R4	80.66	87.66	84.16
S1	92.33	94.0	93.16
S2	89.33	94.36	91.85
ZB-SK-5	85.04	86.66	85.85
Control	85.0	83.66	84.33
	87.06	89.89	

CD (5%)

A-NS

B-7.15

AB-NS

SE-3.4

A - Nitrogen levels

B - Azorhizobium isolates

Figure 46: Effect of inoculation of *Azorhizobium* isolates on plant height of rice cv PR-115 under field conditions

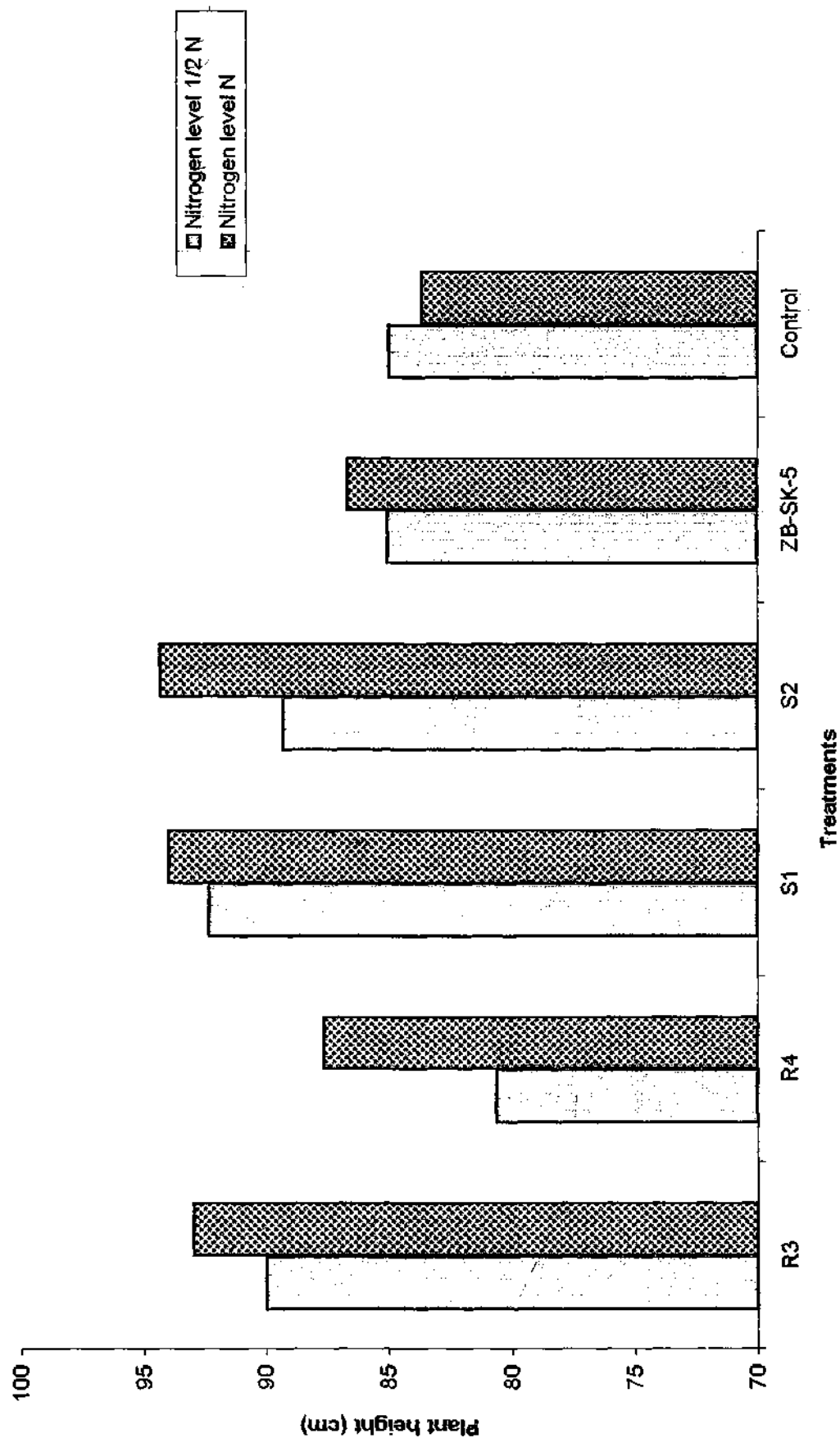


Table 51: Effect of inoculation of *Azorhizobium* isolates on tiller number of rice cv PR-115 under field conditions

Treatment	Tiller number		Mean
	1/2 N	N	
R3	11.66	13.33	12.50
R4	14.00	11.33	12.66
S1	13.00	10.00	11.50
S2	12.33	10.33	11.33
ZB-SK-5	10.33	10.00	10.16
Control	10.66	8.66	9.66
	12.00	10.61	

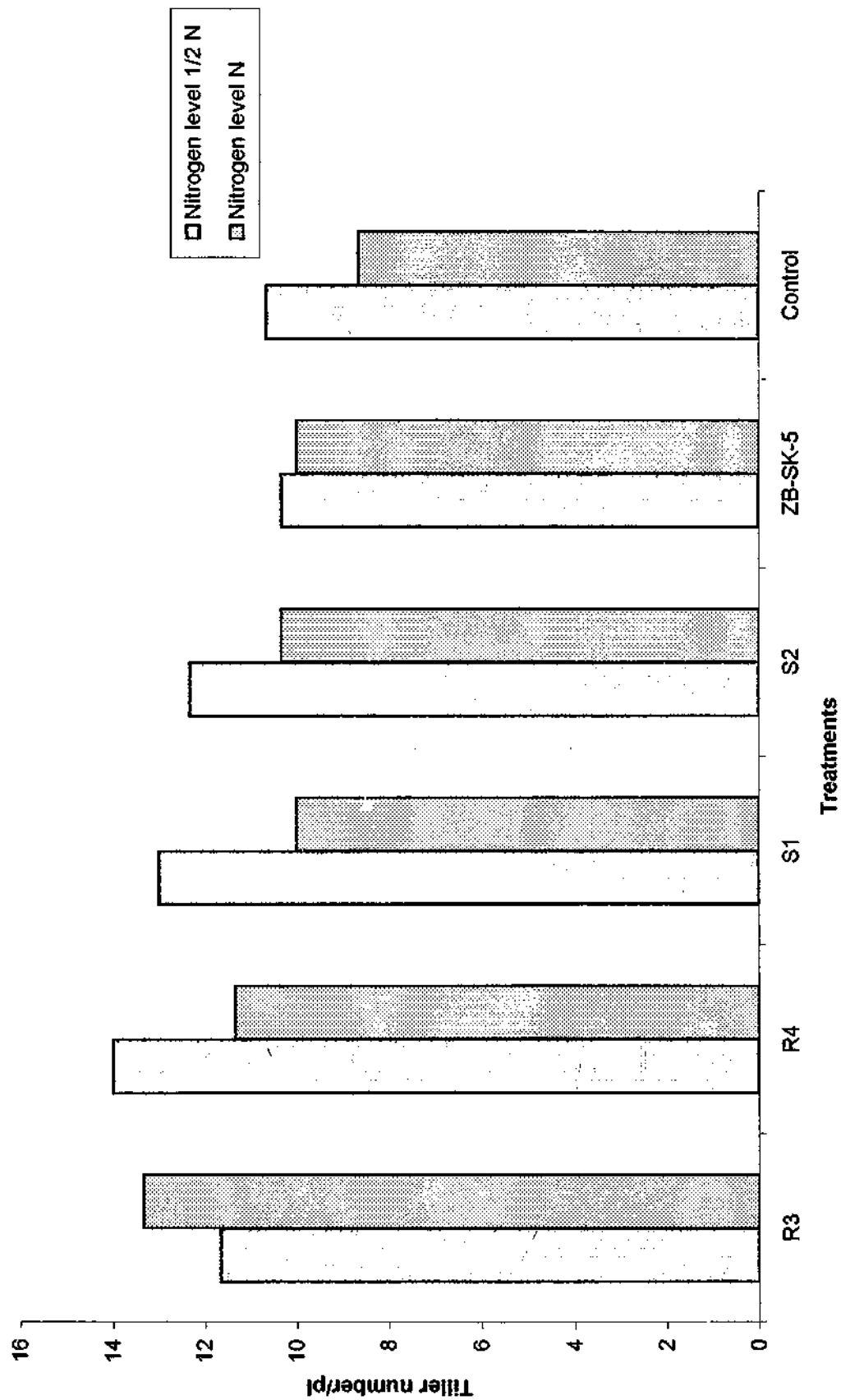
CD (5%) A-1.18 B-2.04 AB-NS

SE -0.986

A - Nitrogen levels

B - *Azorhizobium* isolates

Figure 47: Effect of inoculation of *Azorhizobium* isolates on tiller number of rice cv PR-115 under field conditions



full N plants. Both root isolates performed significantly better over the control while both stem isolates and the reference culture were observed to be statistically at par with the control. The best performer amongst all was recorded as R₄ followed by R₃. Both root isolates performed statistically at par with both stem isolates and significantly better over the reference culture.

4.4.2.3 Panicle Length

All isolates performed statistically at par with each other at both N levels (Table 52 and figure 48). The average panicle length ranged from 21.91-25.45 cm. At half N level all azorhizobial isolates showed no significant difference over the control while all isolates worked significantly better over the control at N full level. On an average all isolates and the reference culture gave significantly higher values over the control. Maximum average panicle length was produced by S₁ followed by the reference culture.

4.4.2.4 Shoot dry weight

Table 53 and figure 49 revealed that no significant difference was occurred among the plants at half N and full N levels. Though half N level plants showed a nonsignificant increase over N full plants. All isolates were found to be statistically at par with the control except S₁ and R₄ that performed significantly better over the control. R₄ produced maximum shoot dry weight followed by S₁. Incorporation of *S.rostrata* and *A.caulinodans* application recorded higher grain and straw yield (Kannaiyan and Kalidurai, 1995).

Table 52: Effect of inoculation of azorhizobial isolates on panicle length (cm) of rice cv PR-115 under field conditions

Treatment	Panicle length		Mean
	1/2 N	N	
R3	22.5	26.33	24.41
R4	22.83	24.66	23.75
S1	25.25	25.66	25.45
S2	24.83	24.00	24.41
ZB-SK-5	24.50	24.50	24.5
Control	23.50	20.33	21.91
	23.9	24.25	

CD(5%) A-NS B-1.98 AB-2.80

SE-0.954

A - Nitrogen levels

B - Azorhizobium isolates

Figure 48: Effect of inoculation of *Azorhizobium* isolates on Panicle length of rice cv PR-115 under field conditions

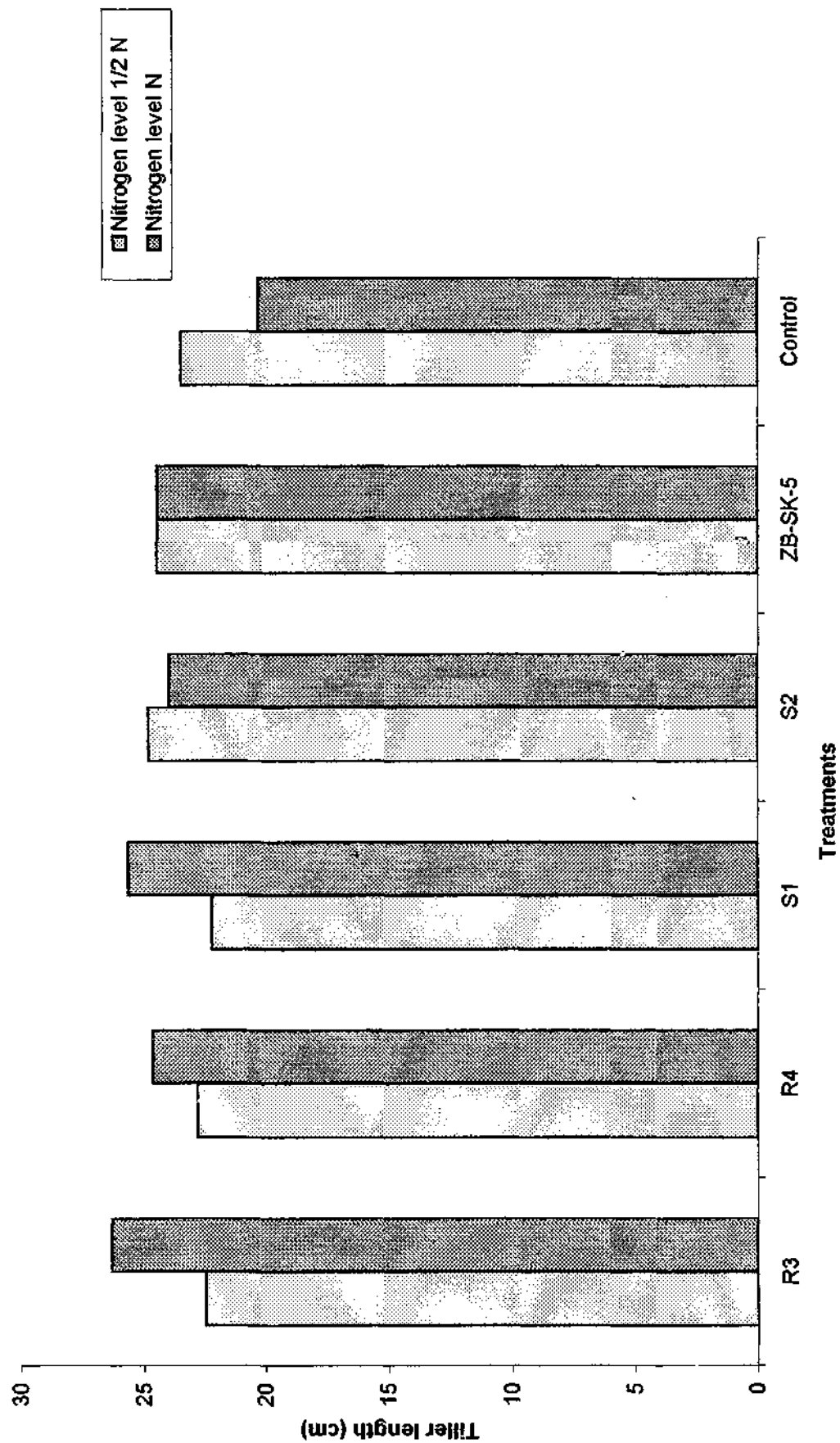


Table 53: Effect of inoculation of azorhizobial isolates on dry weight (g/pl) of rice cv PR-115 under field conditions

Treatment	Dry weight		Mean
	1/2 N	N	
R3	37.7	45.00	41.35
R4	59.46	54.16	56.81
S1	56.16	43.91	50.0
S2	45.46	48.53	47.0
ZB-SK-5	50.14	44.00	47.07
Control	43.33	41.40	42.36
	48.71	46.16	

CD (5%)

A-NS

B-5.69

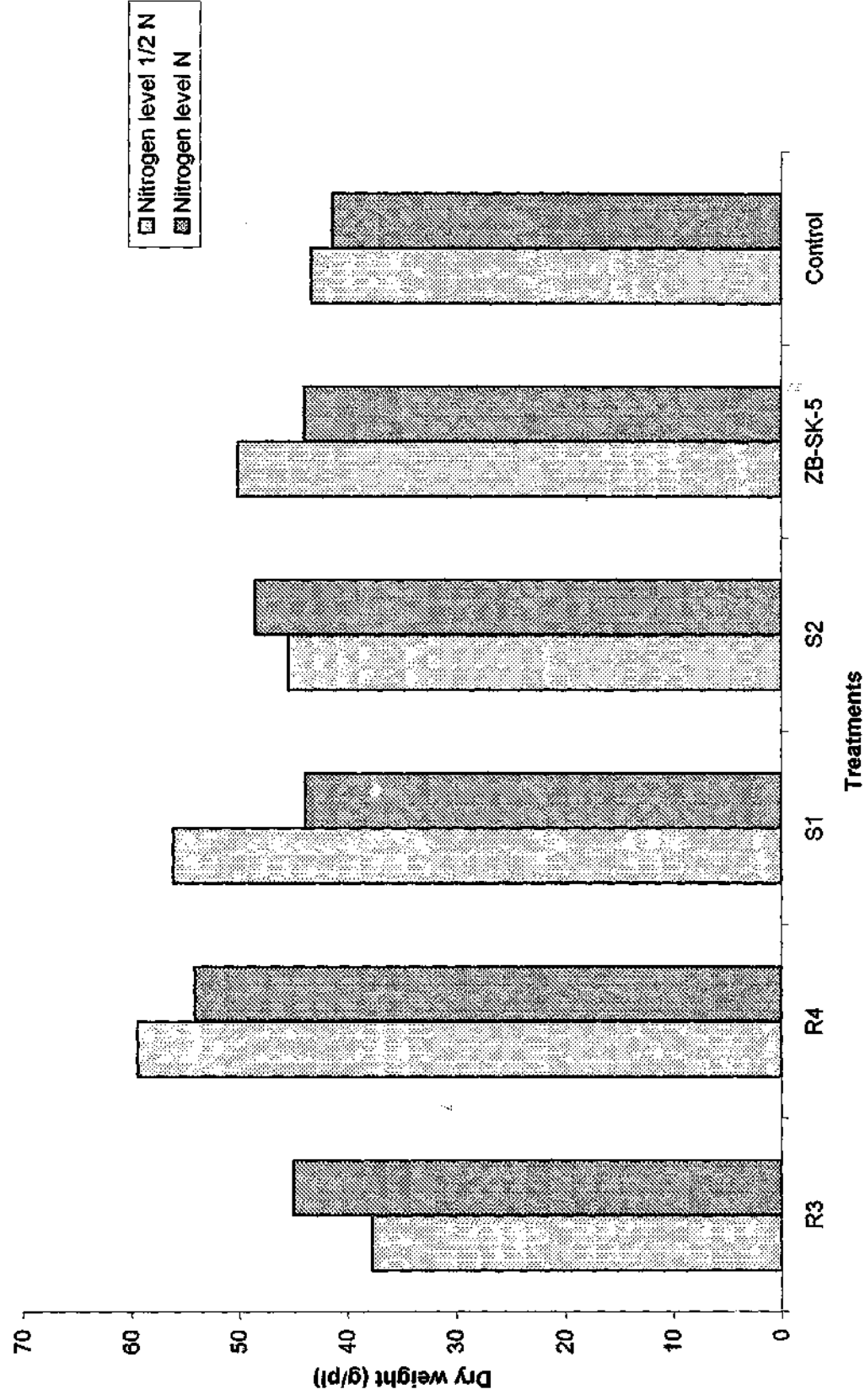
AB-NS

SE-2.74

A - Nitrogen levels

B - Azorhizobium isolates

Figure 49: Effect of inoculation of *Azorhizobium* isolates on dry weight of rice cv PR-115 under field conditions



4.4.2.5 Plant grain weight

The average grain weight ranged from 17.23-29.04 g (Table 54 and figure 50). All isolates showed a nonsignificant increase in half N and N full levels. Among isolates R₄ and S₁ produced significantly higher grain weight over the control while the rest of the isolates gave a nonsignificant increase in grain weight over the control. The best performer amongst all was R₄ followed by S₁. The reference culture was found to be statistically at par with both root and stem isolates. Kalidurai and Kannaiyan (1995) have reported that incorporation of *Sesbania* sp. with fertilizer nitrogen significantly increased the nitrogen uptake, grain and straw yield. Thus the results are similar to the reference reported.

4.4.1.6 Root dry weight

No significant difference was observed between half N and full N levels (Table 55 and figure 51). The root dry weight was observed to vary from 0.582-0.950g. The best performer amongst all was S₁ followed by the reference strain. All azorhizobial isolates performed significantly better over the control except R₃ and S₂ that were found to be statistically at par with the control. Among root isolates R₄ gave significantly better results over R₃ while among stem isolates S₁ performed significantly better over S₂. The reference culture gave significantly more root dry weight over R₃ but performed statistically at par with the rest of the isolates. The root dry weight could have increased due to increased nitrogen uptake by the rice plant on incorporation of *S.rostrata*. The changed root morphology is showed in plate 24.

Table 54: Effect of inoculation of azorhizobial isolates on grain weight (g) of rice cv PR-115 under field conditions

Treatment	Grain weight		Mean
	1/2N	N	
R3	20.50	20.50	20.5
R4	27.59	30.50	29.04
S1	30.95	26.40	28.67
S2	23.30	23.20	23.11
ZB-SK-5	23.64	22.00	22.82
Control	18.95	15.51	17.23
	24.1	23.1	

CD (5%)

A-NS

B-8.23

AB-NS

SE-3.97

A - Nitrogen levels

B - Azorhizobium isolates

Figure 50: Effect of inoculation of *Azorhizobium* isolates on grain weight of rice cv PR-115 under field conditions

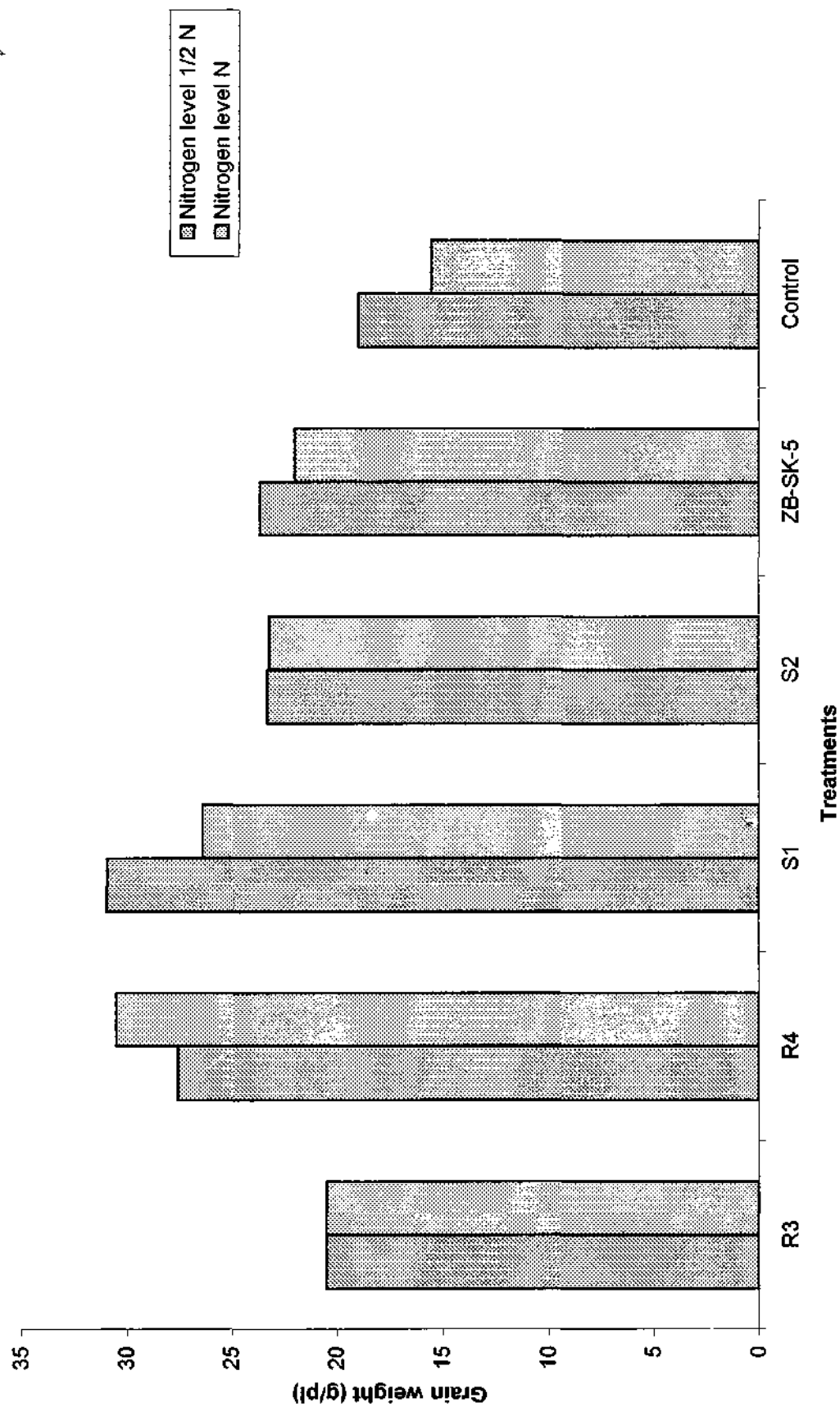


Table 55: Effect of inoculation of azorhizobial isolates on root dry weight (g/pl) of rice cv PR-115 under field conditions

Treatment	Root dry weight		Mean
	1/2 N	N	
R3	0.678	0.502	0.590
R4	0.949	0.682	0.815
S1	0.948	0.952	0.95
S2	0.685	0.675	0.680
ZB-SK-5	0.846	0.80	0.824
Control	0.57	0.595	0.582
	0.779	0.701	

CD (5%) A-NS B-0.155 AB-NS

SE-0.083

A - Nitrogen levels

B - Azorhizobium isolates

Figure 51: Effect of inoculation of *Azorhizobium* Isolates on root dry weight of rice cv PR-115 under field conditions

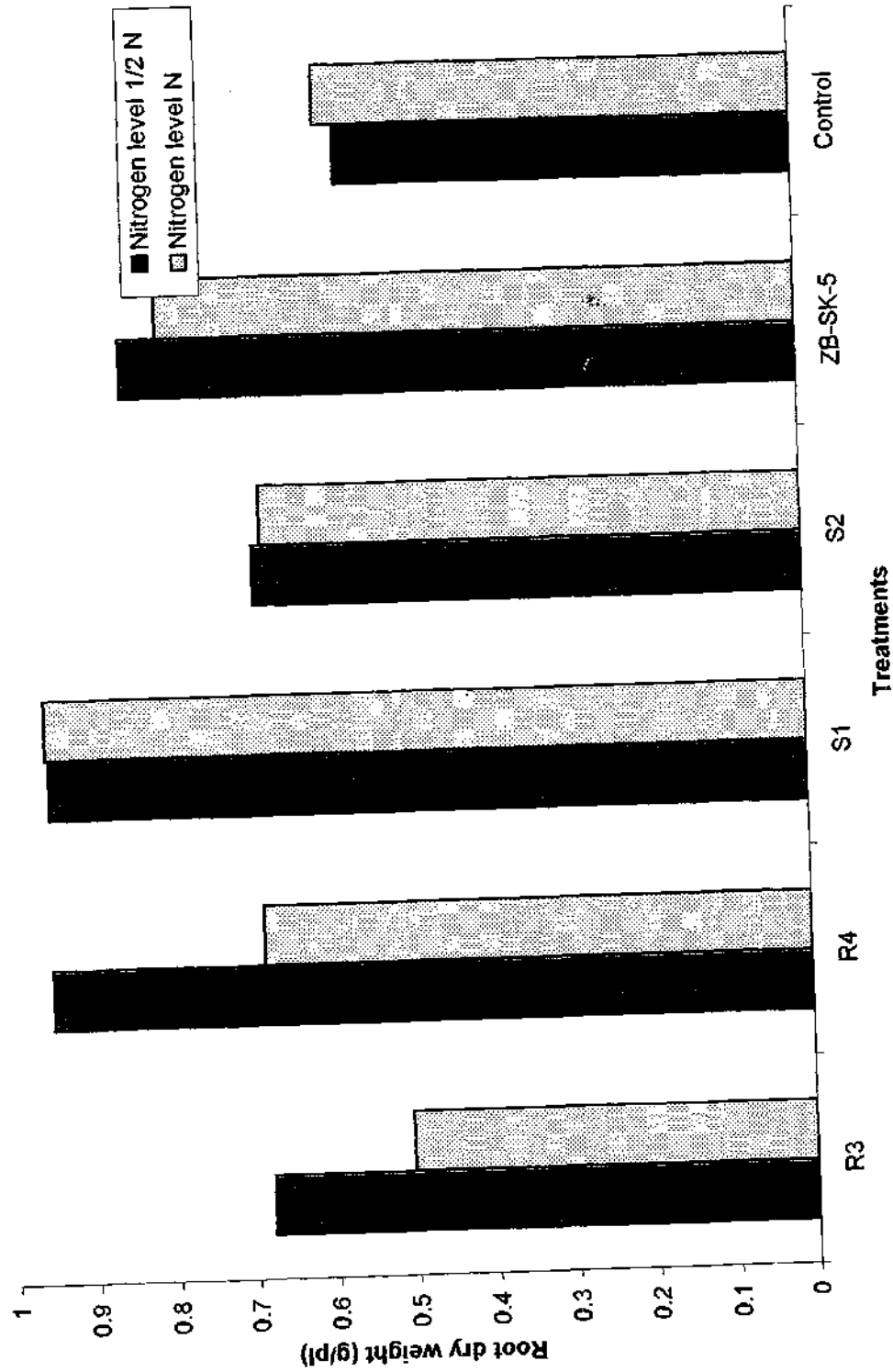


Plate 23: Effect of inoculation of *A. caulinodans* isolates on rice cv PR-115 under field conditions

Plate 24: Modified lateral root structures observed in rice cv PR-115 under field conditions



Plate 23



Plate 24

4.4.2.7 Paranodule number

The results were presented in Table 56 and figure 52 revealed that the plants in *Sesbania* buried plots produced significantly more number of paranodes over N full level i.e. no *Sesbania* buried plots (Plate 25). The average paranodule number ranged from 2.65-17.47. All isolates except R₄ and A.c. ZB-SK-5 (which were observed to be statistically at par with the control) performed significantly better over the control. R₃ gave significantly better results over R₄. S₁ stem isolate performed significantly better over S₂. Maximum number of paranodes were recorded by S₁ followed by R₃. All isolates except R₄ performed significantly better over the reference culture. The paranodule number of plants of the *Sesbania* incorporated plots increases could be presumed due to the increase in number of the azorhizobia in the plot as well as due to increased activity of the cellulytic fungi and bacteria.

4.4.1.8 Root nitrogenase activity

Table 57 and figure 53 revealed the range of nM acetylene produced to be from 8.8-15.49 nM C₂ H₂ plant⁻¹ h⁻¹. The difference between all isolates was found to be nonsignificant. All isolates showed a nonsignificant increase in nitrogenase activity over the control. The half N level gave significantly higher nitrogenase activity over the control. The maximum nitrogenase activity was recorded by S₁ followed by S₂ at both the N levels. Van-Nieuwenhave *et al* (2000) reported low nitrogenase activity of the rice roots inoculated with the *Azorhizobium caulinodans* under wetland conditions.

Table 56: Effect of inoculation of azorhizobial isolates on paranodule number of rice cv PR-115 under field conditions

Treatment	Paranodule no.		Mean
	1/2N	N	
R3	25.0	1.0	12.83
R4	8.0	-	4.00
S1	26.0	8.92	17.47
S2	14.5	2.05	8.27
ZB-SK-5	6.4	1.52	3.47
Control	5.3	-	2.65
	14.15	2.08	

CD(5%) A-2.08 B-3.60 AB-5.10

SE-1.739

A - Nitrogen levels

B - Azorhizobium isolates

Figure 52: Effect of inoculation of *Azorhizobium* isolates on paranodule formation in rice cv PR-115 under field conditions

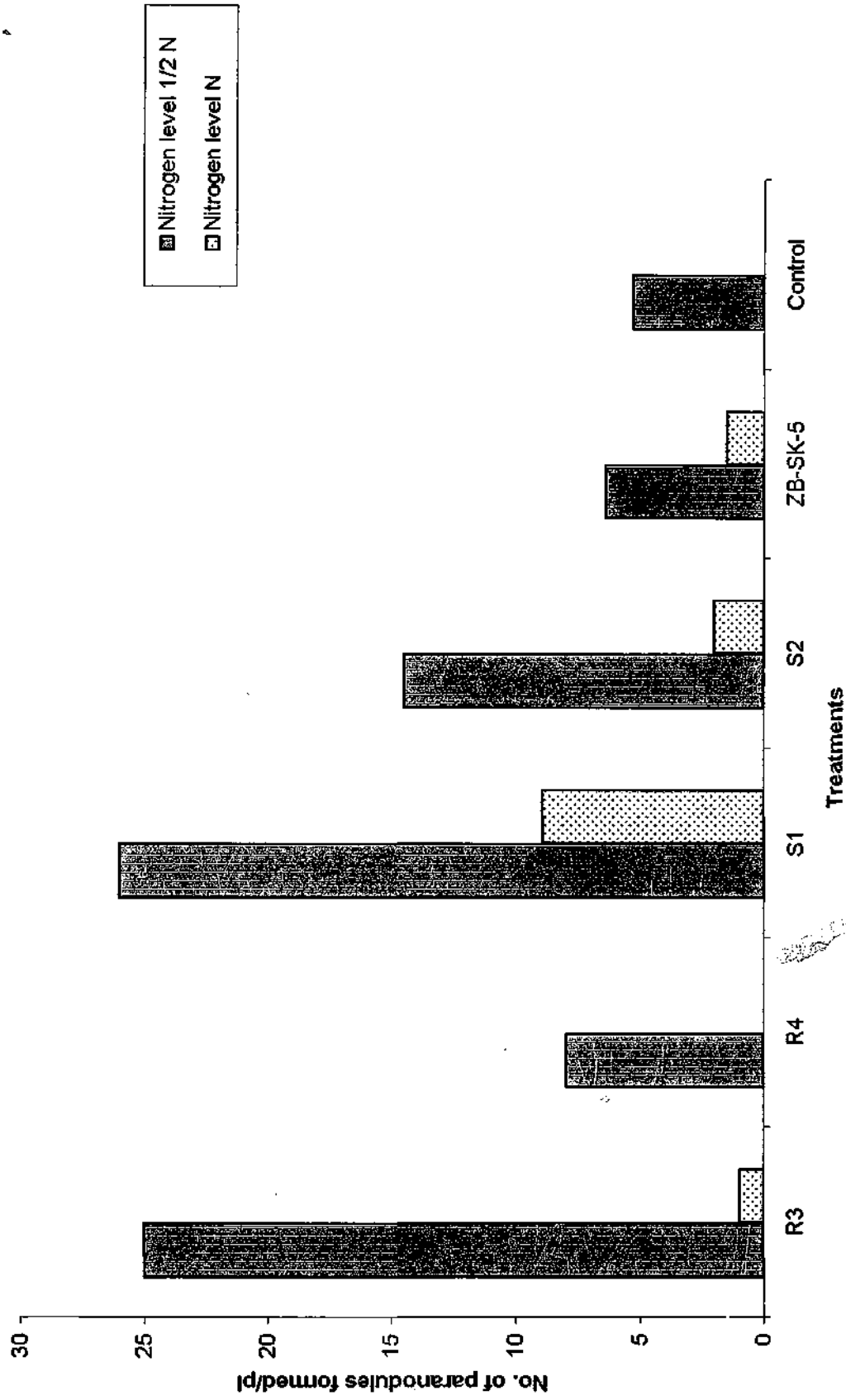


Table 57: Effect of inoculation of azorhizobial isolates on root nitrogenase activity (nM/plant/hr) of rice cv PR-115 under field conditions

Treatment	Nitrogenase activity		Mean
	1/2 N	N	
R3	13.78	9.0	7.76
R4	17.03	7.58	12.3
S1	18.56	12.68	15.49
S2	16.93	9.54	13.24
ZB-SK-5	15.23	8.40	9.27
Control	12.27	5.36	8.8
	13.55	8.73	

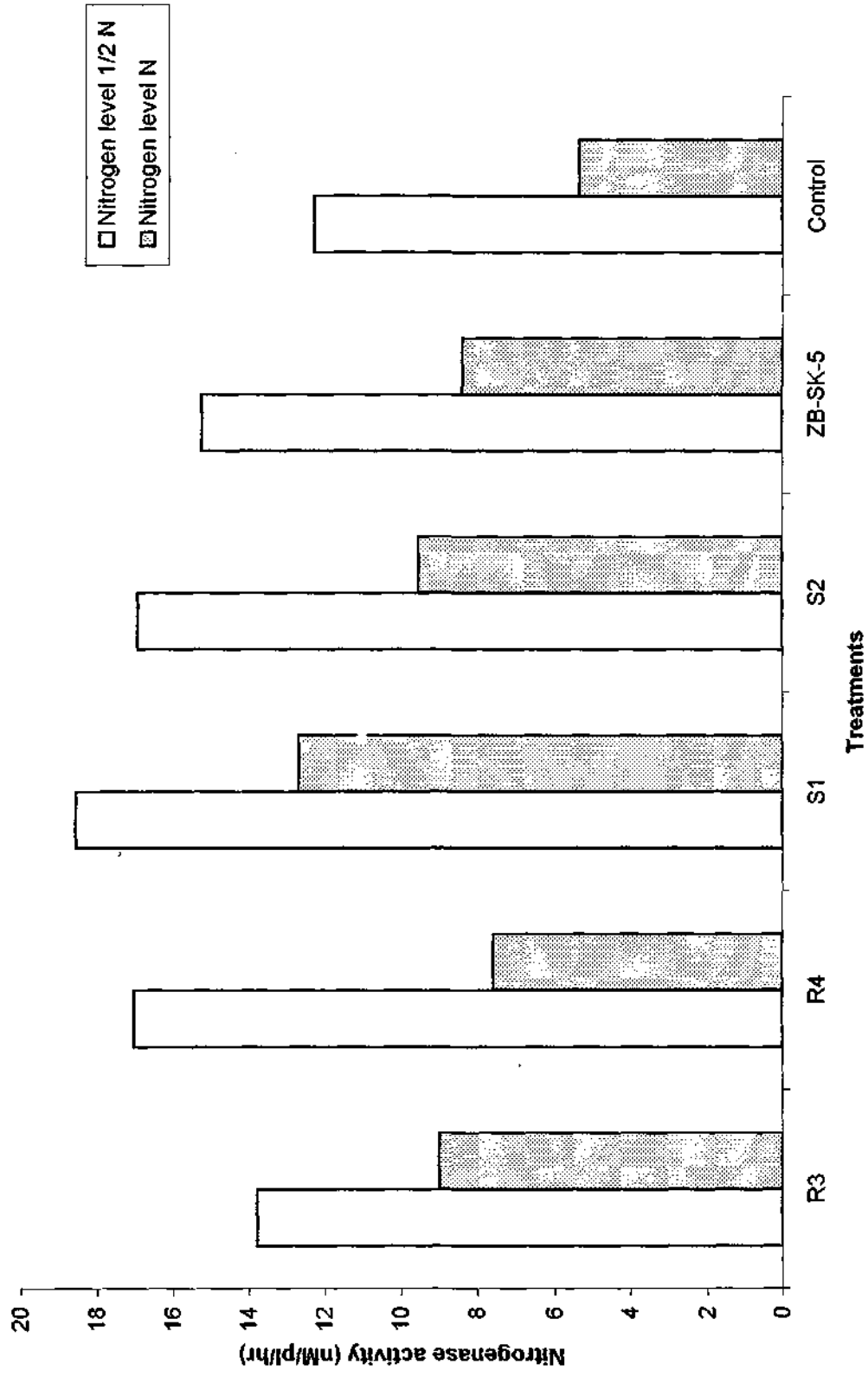
CD(5%) A-4.28 B-NS AB-NS

SE-3.57

A - Nitrogen levels

B - Azorhizobium isolates

Figure 53: Effect of inoculation of *Azorhizobium* isolates on root nitrogenase activity of rice cv PR-115 under field conditions



4.4.2.9 Percent establishment of the inoculated isolates

The isolates were tested by using intrinsic antibiotic markers for their ability to establish in the rhizosphere of the rice roots and Table 58 and figure 54 revealed that the competitiveness of various isolates ranged from 18.5-37%. All isolates were found to be more competitive over the native flora. The establishment of isolates was observed to be significantly better in half N level over N full level. Similarly, Van-Nieuwenhave *et al* (2000) reported establishment of *Azorhizobium caulinodans* in the rhizosphere of wetland rice.

4.4.2.10 Yield

Table 59 and figure 55 revealed the average range of yield from 41.05-50.96 Q/ha. The difference in yield of both N levels was found to be nonsignificant thus indicating that yield was irrespective of the N added. All isolates produced significantly higher yield over the control while the difference between the isolates was observed to be nonsignificant. The maximum yield was produced by S₁ followed by R₃. Incorporation of *S.rostrata* at 20 t/ha significantly increased the grain yield of rice (Chitra and Kannaiyan, 1995) due to significantly increased nitrogen uptake by the rice plants (Kalidurai and Kannaiyan, 1991). Similarly Dreyfus *et al* (1985) observed that the incorporation of *S.rostrata* at 20 t/ha increased the yield of rice by 53% over the fertilizer nitrogen at 60 Kg N ha⁻¹.

4.4.2.11 Histological studies

The study of the microtomic sections revealed increased accumulation of bacterial cells on the surface of the roots of the *Sesbania* buried treatments in

Table 58: Percent establishment of Azorhizobial isolates in rice cv PR-115 under field conditions

Trèatment	% establishment		Mean
	1/2N	N	
R3	33.3	18.5	25.9
R4	33.3	22.2	27.76
S1	37	25.9	31.45
S2	29.6	25.9	27.75
ZB-SK-5	25.9	22.2	24.05
Control	-	-	-
	26.52	19.11	

CD(5%) A-3.75 B-6.49 AB-NS

SE-3.13

A - Nitrogen levels

B - Azorhizobium isolates

Table 59: Effect of inoculation of azorhizobial isolates on yield (q/ha) of rice cv PR-115 under field conditions

Treatment	Yield		Mean
	1/2 N	N	
R3	51.36	48.32	49.72
R4	46.73	49.99	48.36
S1	52.0	49.88	50.96
S2	52.0	47.33	49.66
ZB-SK-5	47.37	49.05	48.21
Control	40.88	41.22	41.05
	49.54	46.16	

CD(5%)

A-NS

B-4.64

AB-NS

SE-2.23

A - Nitrogen levels

B - Azorhizobium isolates

Figure 54: Effect of Inoculation of *Azorhizobium* isolates on yield of rice cv PR-115 under field conditions

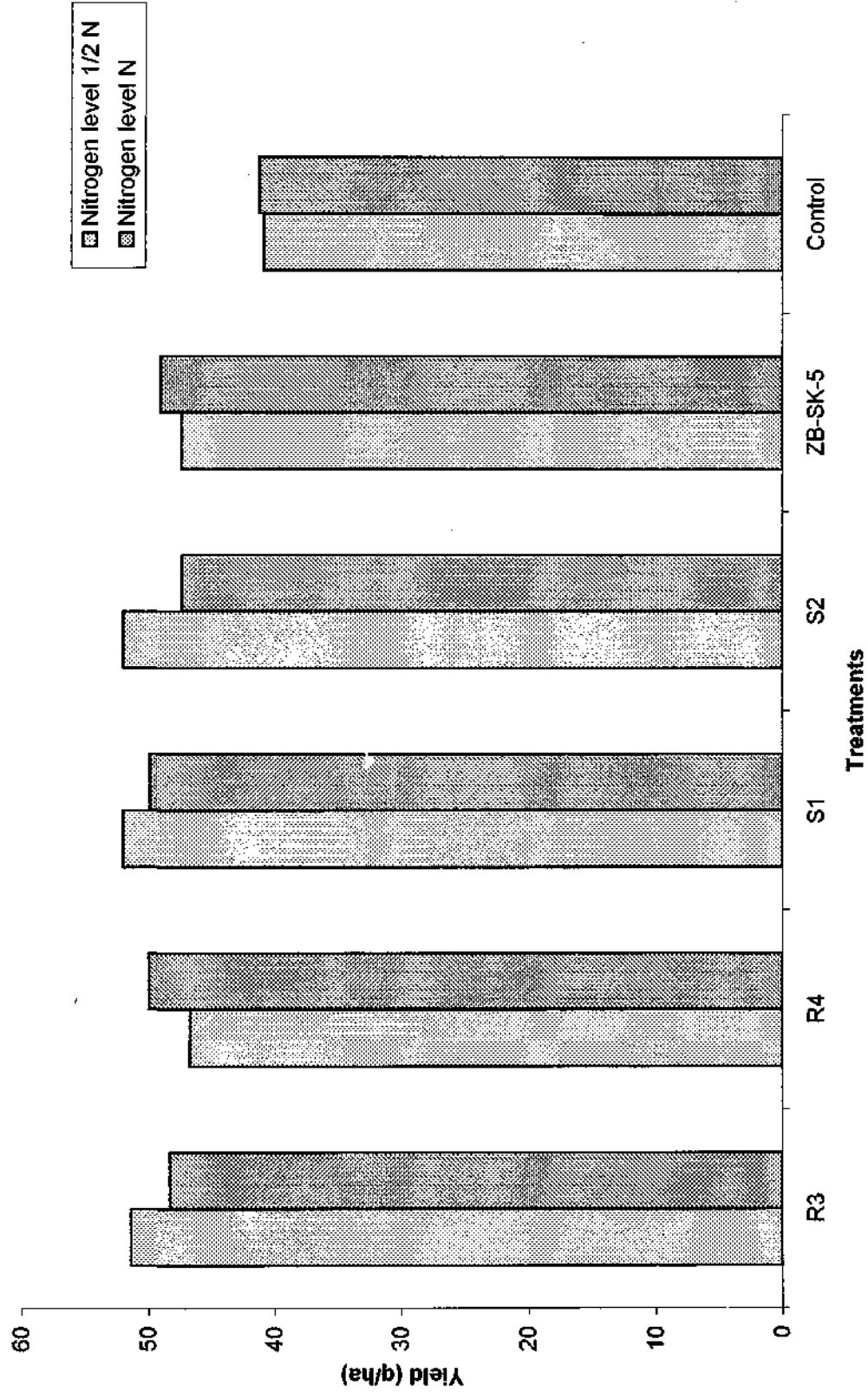


Figure 55: Percent establishment of inoculated *Azorhizobium* isolates in rice cv PR-115 under field conditions

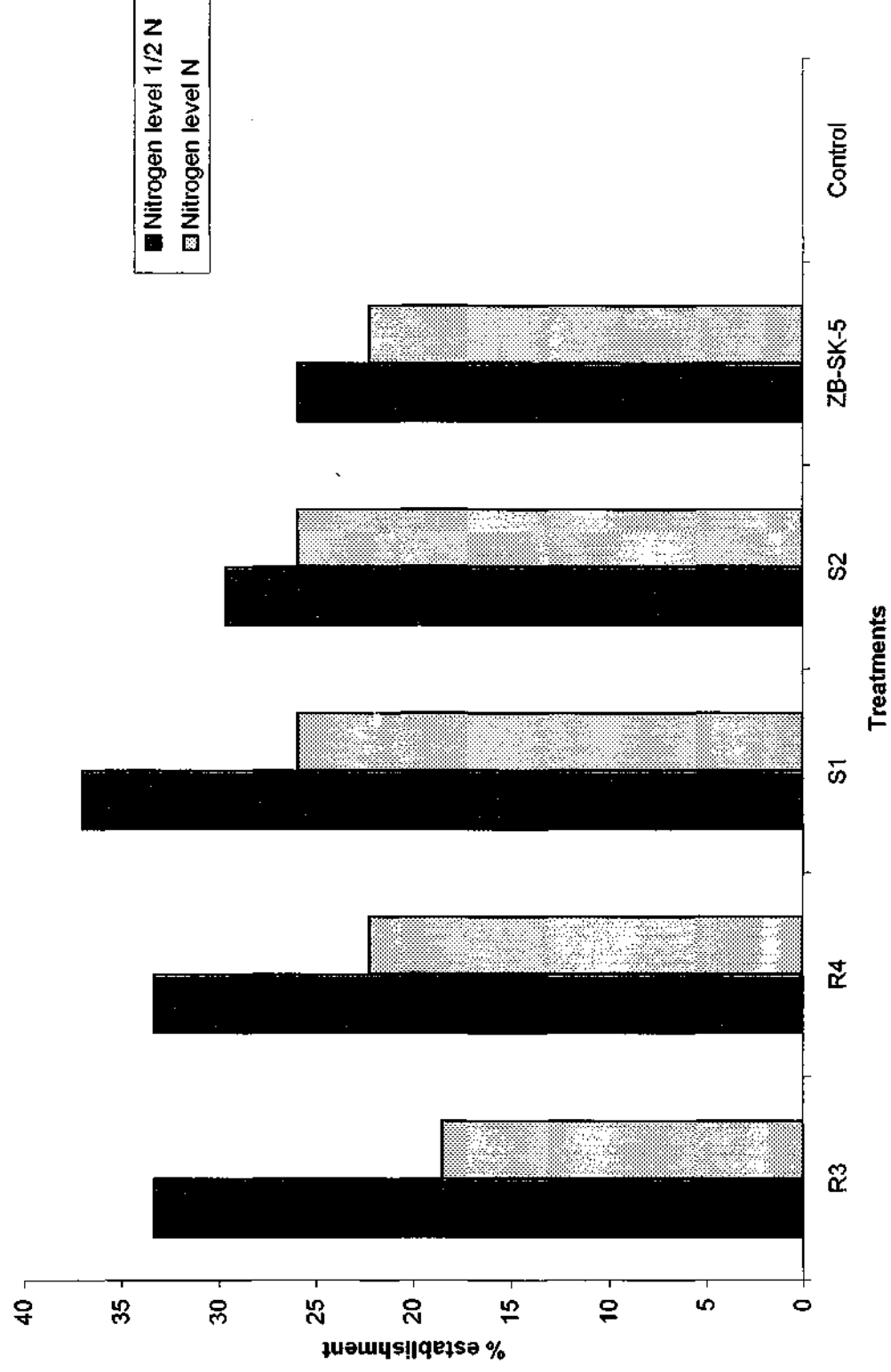


Plate 25: Close view of the swollen structures observed in rice under field conditions

Plate 26: Stained microtomic section of rice roots inoculated by *A. caulinodans* isolates under field conditions



Plate 25

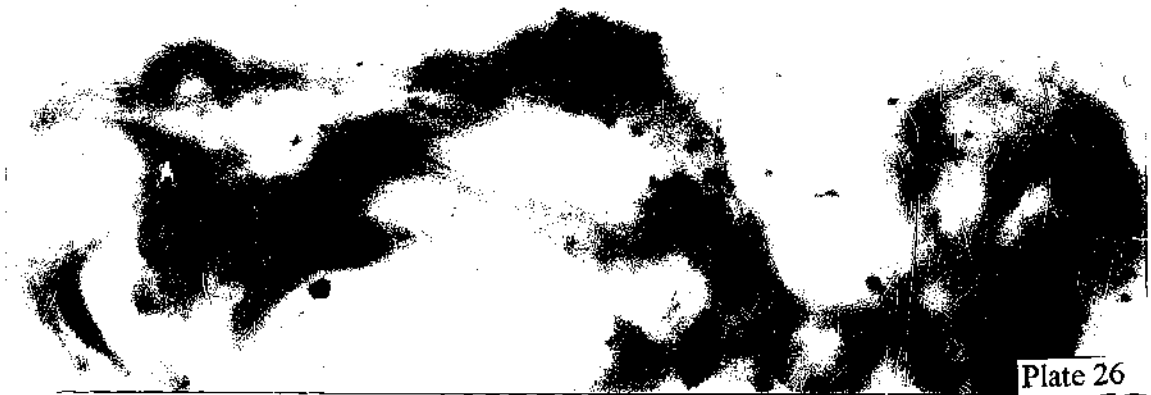


Plate 26

comparison to the control roots (Plate 26). The former sections also showed rare bacterial encroachment inside the rice roots below the root epidermal cells.

Chapter-V

SUMMARY

A diversity of nitrogen fixing bacteria remain naturally associated with field grown rice (Bally *et al*, 1983; Stoltzfus *et al*, 1997). Indirectly rice is able to utilize a basal level of fixed nitrogen as a source of it's nitrogen nutrition from the nitrogen fixing activities of diazotrophs in it's agronomic ecosystem (Yanni, 1991; Roger and Ladha, 1992; Ramasamy and Bal, 1986). Among diazotrophs *Azorhizobium caulinodans* that forms root and stem nodules on *Sesbania rostrata*, a tropical legume has the potential of contributing towards rice production due to it's ability to invade lateral roots by crack entry. 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 and thus such practices could enhance the rice productivity along with no or minimal environmental pollution extending a great step towards economically low cost sustainable agriculture (Swensen and Mullis, 1997; Yadav *et al*, 2000). The results of present study are summarized as below:

1. A total of six *Azorhizobium caulinodans* isolates were isolated from root as well as stem nodules of *Sesbania rostrata*. The isolates identified as *A. caulinodans* on examination for Gram reaction, motility, growth and colony characteristics on yeast extract mannitol agar supplemented with Congo red dye, Bromothymol blue dye and Lactose. The azorhizobial

isolates were further tested for growth in Hofer's medium. Most of the isolates were found to be fast growers and acid producers. Both stem isolates were fast growers while among root isolates R₄ was found to be slow grower.

2. The nitrogen fixing potential of isolates were measured by the acetylene reduction assay and the maximum in vitro nitrogenase activity was recorded by the stem isolate S₁ followed by S₂, R₄, R₃, R₂ and R₁. The reference strain ZB-SK-5 reduced 776.25 nM acetylene h⁻¹mg⁻¹ protein which was lesser than both the stem isolates. Based on nitrogenase activity R₃, R₄, S₁ and S₂ were selected as the better performers and were used to study induction of nodule like structures and invasion of azorhizobia in the roots of rice cv PR-115.
3. The intrinsic antibiotic resistance spectra of isolates were worked out and variations among isolates were observed regarding the ability to resist particular antibiotic. The stem isolates showed resistance to most of the antibiotics used while the root isolates were found to be resistant to ampicillin, chloramphenicol and neomycin. R₄ was found to be sensitive to all the antibiotics used at 100 µg ml⁻¹ working concentration.
4. **Induction of nodulation in rice by *A.caulinodans* along with phytohormones and signal compounds laboratory conditions.**

2,4-D

The effect of inoculation of *A.caulinodans* isolates with 2,4-D on the growth and paranodule formation in rice was studied and results proposed that inoculation of S₁ isolate with 2,4-D at 3.0 ppm concentration produced better

results than other isolates. This treatment decreased root length and increased the shoot length as well as nitrogenase activity compared to control. The maximum lateral rootlet number, fresh and dry weight were recorded by S₁ isolate at 1.5 ppm concentration of 2,4-D.

NAA

Treatment with NAA resulted in decrease in root and shoot length parameters except paranodule number and nitrogenase activity that were observed to increase with increase in NAA concentration. NAA at 3.0 ppm concentration with S₁ isolate produced better results over other concentrations as well as rest of isolates except effect on the paranodule number and nitrogenase activity that were found to be maximum in seedlings treated with NAA at 7.0 ppm with S₁ isolate.

Naringenin

An increase in root length, shoot length, number of paranodes, lateral rootlet number and nitrogenase activity was observed in rice seedlings treated with naringenin at 10⁻⁵ M concentration with S₁ isolate. 10⁻⁵ M concentration of naringenin produced better results over rest of the 2 concentrations among all the isolates as well as over the uninoculated control.

Acetosyringone

Inoculation with R₄ at 400 ppm concentration of acetosyringone resulted in increase in lateral rootlet number, nitrogenase activity, paranodule number, shoot length and fresh and dry weight of shoot while a decrease in root length was observed in the above treatment. Acetosyringone at 400 ppm produced better results over the other concentration.

Histological studies and reisolation of *A.caulinodans*

Histological analysis of the stained microtomic sections showed presence of bacteria on the surface and inside the cortex of rice roots with 2,4-D treated roots showing more number of bacteria. Reisolation was carried out from the suspension of crushed nodule like structures and lateral rootlets produced in the rice roots treated with 2,4-D, NAA, Naringenin and Acetosyringone and inoculated with *A.caulinodans* isolates. The results confirmed the presence of *A.caulinodans* inside the nodule like structures.

5. Screening of *Azorhizobium* isolates

- a) Pot trial for *Sesbania rostrata* was carried out by selecting all the six isolates (4 root +2 stem) along with reference strain *A.caulinodans* ZB-SK-5 that were seed inoculated for the above crop in the pots. Different parameters as plant height, fresh weight, root biomass, nodulation status, nitrogenase activity and establishment of inoculated isolates were studied. *Azorhizobium* inoculation resulted in increase in all parameters studied with S₂ isolate being the best performer amongst all isolates followed by S₁ under pot conditions. All the isolates were found to be established in the soil however the extent of establishment of isolates varied from 33.3 – 88.8% with S₁ showing maximum establishment followed by S₂ whereas R₁ was found to be least established.
- b) In field trial for *Sesbania rostrata* all the 6 isolates and the reference strain *A.caulinodans* ZB-SK-5 were seed inoculated and all parameters studied in pot trial were recorded. The field trial results revealed an increase in growth characters and yield in *S.rostrata* by *A.caulinodans* isolates inoculation. S₁ isolate performed best for all parameters except nodule number, fresh and dry weight. The local isolates competed well with the native flora and established to form nodules with % establishment ranging from 33.3-77.7 %.

6. Effect of burial of *S.rostrata* and *A.caulinodans* on paranodulation, nitrogen economy and yield of rice

In the field trial for rice cv PR-115 the isolates screened in the previous experiments were applied to *Sesbania*. As in the previous field and pot trial of *Sesbania rostrata* S₁ performed best amongst all in production of fresh biomass, root and shoot nitrogenase activity. While S₂ performed best in parameters as plant height, root and stem nodule number, fresh weight and dry weight. All isolates were found to establish in rhizosphere soil but the extent of competitiveness ranged from 59.2-81.4%. Maximum establishment values were observed in S₁ followed by S₂ while the reference strain showed least establishment.

Later the *S.rostrata* plants were buried 45 DAS and resulted in increase in growth and yield as well as caused increase in paranodulation and nitrogenase activity due to the crack entrance of the azorhizobia inside the rice roots with S₁ being the best performer for plant height, panicle length and yield at both nitrogen levels and paranodule formation and root nitrogenase activity for plants having half-N dose and R₄ for tiller number, root and shoot dry weight and grain weight at both N-levels.

The histological studies revealed the increased aggregation of Azorhizobia on the surface of the roots of plants of *S.rostrata* buried plots than the control roots. All isolates were found to be established in the rice rhizosphere but the extent ranged from 18.5-37%. The establishment of isolates was significantly more in half N level than N full level.

Chapter-VII

CONCLUSIONS

A.caulinodans, a stem-nodulating diazotroph of *S.rostrata* can paranodulate non-legumes as rice due to invasion via crack entry. In present study six isolates isolated from root and stem nodules showed increase in biomass, nitrogenase activity and grain yield in *Sesbania rostrata* plants both under pot and field conditions with most of the isolates excelling in performance over the standard culture. This calls for an eminent urge to accelerate efforts for developing potential technologies regarding use of local isolates.

Four isolates of *A.caulinodans* were used to induce nodulation in rice cv PR-115 along with a reference strain in presence of phytohormones (2,4-D and NAA) and signal compounds (Naringenin and Acetosyringone) at very low working concentrations. *Azorhizobium* isolates inoculation caused nodule induction and nitrogen fixation that resulted in increase in biomass of rice for phytohormones and naringenin treatments with S₁ isolate being the best performer while for the acetosyringone treatment R₄ gave best results. The latter treatment showed paranodulation and intercellular incorporation that suggests a link between *Azorhizobia* and *Agrobacteria* as the *Agrobacterium tumefaciens* uses acetosyringone as a potent signal compound.

Histological studies confirmed accumulation in increased number on surface of rice root epidermal cells and intercellular incorporation of the bacterial

cells in the rice cortical cells with 2,4-D treated roots giving better incorporation in the cortex.

The isolates also performed well on rice under field conditions with encouraging results regarding paranodule induction, nitrogenase activity and the yield of rice. The expression of nitrogenase activity in cell cultures and formation of paranodes in *Sesbania* supplemented plots showed that the contribution in terms of nitrogen economy and increased rice yield of *Sesbania* is not only because of biomass or nutrients being supplied by the *Sesbania* plant but also due to the nodulation and nitrogen fixation of the rice crop itself. This raises the possibility of using the *Azorhizobium* as a future biofertilizer for the non-legume crops as only *A.caulinodans* isolate inoculation increases the growth and yield characters of the rice.

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