EVALUATION OF AZIDE RESISTANT MUTANTS OF AZOSPIRILLUM ON MAIZE

Thesis submitted to the
University of Agricultural Sciences, Dharwad
In partial fulfillment of the requirements for the
Degree of

MASTER OF SCIENCE (AGRICULTURE)
IN
AGRICULTURAL MICROBIOLOGY

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NOVEMBER,2006

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I. INTRODUCTION

The advancing crop production technology, development of high fertilizer responsive crop varieties and pressure for intensive farming demand huge inputs of which, nitrogenous fertilizers take a major share. The ever increasing and exploding population, together with the hiking price of petroleum and petroleum based products tightens the global situation to such a rigorous extent that the nitrogenous fertilizers have not only become costlier but also quite insufficient to meet the demands of the farming community as a whole. Convincing evidences have pointed out that biological nitrogen fixation (BNF) requiring a relatively simple and low cost technology easy to execute and largely of renewable sources of energy has a tremendous role to play in the immediate future of agriculture, especially in the developing and underdeveloped countries.

Global gains of annual terrestrial nitrogen fixation range from 139 to 170 metric tones with symbiotic nitrogen fixation in arable farming accounting for 35 to 44 metric tones nitrogen and another 45 metric tones from permanent pastures and rest from non-symbiotic nitrogen fixation. In comparison only 91 metric tones of fertilizer N was globally manufactured, which is only 53-65 per cent of biological fixed N.

Among the nitrogen fixing microorganisms symbiotic group of bacteria viz., *Rhizobium* and *Bradyrhizobium* and non-symbiotic including associative symbiotic group viz., *Azotobacter* and *Azospirillum* are the dominant forms.

As early as 1925, Beijerinck reported this bacterium as *Spirillum lipoferum*. Later, Tarrand *et al.* (1978) renamed the organism as *Azospirillum* (Azote means nitrogen) due to nitrogen fixing capacity and classified all the strains of *Azospirillum* into two groups : *Azospirillum brasilense* and *A. lipoferum*. Dobereiner *et al.* (1981) reported that possession of C4 – dicarboxylic acid pathway of photosynthesis by tropical grasses favours the establishment of nitrogen fixation in the roots because of the ability of these plants to use the intense radiation in tropics efficiently. By the use of *Azospirillum* as inoculant, economy of 20-30 kg N/ha equivalent could be achieved in crops like maize, sorghum, barley, wheat, sugarcane and other millets (Ganguly and Manna, 1999). Besides, nitrogen fixation it also produces growth harmones such as auxins, gibberellins and cytokinins (Yadav *et al.*, 1989).

Maize (*Zea mays* L.) a new world graminaceous crop, is considered as king of crops and queen of cereals. Globally maize is the second most important cereal grain after wheat (if we take into consideration statistics of dehusked edible rice instead of paddy with husk). It has a great world wide significance as human food, animal feed and as a source of large number of industrial products. The diversity of environments under which maize grown is unmatched by any other crop as the expansion of maize to new areas and environments still continues. The renowned noble laureate Dr. Norman E. Borlaug believes that “after the last two decades saw the revolution in rice and wheat, the next few decades will be known as maize era (Patil *et al.*, 2000).

In India, at present, about 35 per cent of maize produced is used for human consumption 25 per cent each in poultry and cattle feed and 15 per cent in food processing and other industries.

In Karnataka, maize occupies an area of 6.9 lakh ha with production of 21.4 lakh tones and the average productivity is about 5.4 tonnes per hectare (Anon., 2003a). The important maize growing districts in Karnataka are Belgaum, Bijapur, Bangalore, Dharwad, Kolar, Mysore and Tumkur. It is grown under irrigated conditions mainly in the commands of Malaprabha, Ghataprabha and Tungabhadra projects of North Karnataka.

The present hike in the price of chemical fertilizers has compelled the Indian farmers to resort to imbalanced nutrition in favour of nitrogen for crops and thus reduction in crop yields. At this juncture, there is an urgent need to optimize the cost of nitrogenous fertilizer production and to more towards cost : benefit ratio. By considering the above facts and

figures we can meet part of our required quantity of nitrogen through a ecofriendly mechanism i.e., biological nitrogen fixation.

Mutagenesis is one of the common and simple approaches to improve the useful traits of a bacterium and has successfully been used in case of *Azospirillum* (Jain and Patriquin, 1985; Kolb and Martin, 1985; Christiansen Waniger and Van-Veen, 1991).

It was therefore, thought necessary to develop associative symbiont which possibly reduce the nitrogen requirement particularly in maize by enhancing the nitrogenase activity of *Azospirillum*, through, selection and mutation. Therefore, the resistance against high doses of sodium azide may be used as a strong relation pressure for enhanced activity of nitrogen fixing microorganisms (Ram *et al*., 1978).

Sodium azide is a potent inhibitor of the terminal segment of electron transport chain (Linnett and Bechey, 1979; Vasudeva *et al*., 2003). However, the molecular mechanism underlying the enhanced nitrogen fixing ability of AziR mutants are not known in any of dinitrogen fixers (Sharma *et al*., 1997).

The azide resistant mutants of *Azospirillum* ACD-802 and ACD-701 fixed more amount of nitrogen compared to wild types ACD-8 and ACD-7 respectively (Dayamani, 2003).

By considering the above factors an investigation was carried out to evaluate the Azide resistant mutants of *Azospirillum* for higher nitrogen fixation with the following objectives.

1. To evaluate the influence of inoculation of azide resistant mutants of *Azospirillum* in combination with different levels of nitrogen on growth, nitrogen uptake and yield of maize crop

2. To study the population dynamics of Azide resistant *Azospirillum* in the endorhizosphere and rhizosphere under field condition in maize crop

3. To assess the stability and competitiveness of azide resistant *Azospirillum* mutant strains.

II. REVIEW OF LITERATURE

Atmosphere comprises of 78 per cent of nitrogen gas. Despite its abundance in the atmosphere, the paradise of nature is only to be assimilated by plants unless it is reduced to ammonia by special group of prokaryotic organisms called nitrogen fixers (Subba Rao, 1981). The group of diazotrophs capable of colonizing the roots of non-legumes gained importance and the association has been termed as “associative symbiosis” or “diazotrophic biocoenosis” (Doberenier and Deppolli, 1980).

The most promising organisms capable of colonizing roots in large members and exerting beneficial effects on plants belong to the genes *Azospirillum*. The practical approach to exploit this microorganism has achieved a new dimension. In India studies on *Azospirillum* were primarily initiated early in the 70’s and investigated to greater details subsequently (Subba Rao, 1981 and Wani, 1992).

An analysis of the studies carried out on the interaction of diazotrophic bacteria and plant have shown greater impact on the biological N_2-fixation in cereals (Dobereiner, 1981; Subba Rao, 1986; Balasubramaniam and Kumar, 1989) non leguminous plants (Baldani *et al*., 1997), Oilseeds (Kumar *et al*., 1995), legumes (Tilak *et al*., 1981, Bashan *et al*., 1989), oil palm (Baldani *et al*., 1997), plantation crops (Merina, 1991 and Subba Rao, 1993), mulberry (Santhana Kishnan and Oblisami, 1988) and ornamental crops (Laxmikumari *et al*., 1976; Hemavathi, 1997) and association of diazotrophs have been subjected to detailed study.

2.1 DIAZOTROPHIC BIOCOENOSIS

The rhizocoenosis is the culmination of an association of *Azospirillum* and plant root interaction. This interaction takes place in the rhizosphere or within the root tissue, but no specialized structures comparable to the legumes root nodule induced by *Rhizobium* are formed. The association can therefore, be best described as a more colonization of the rhizosphere, rhizoplane and/or the root interior (Michiels *et al*., 1989).

Ruschel and Vose (1981) suggested the term “diazotrophic biocoenosis” for associative N_2-fixing biological system and “diazotrophic rhizocoenosis” for association of N_2-fixing system in or in the close vicinity to the root.

Azospirillum rhizocoenosis was first reported in the forage grass *Digiteria decumbens*. The rhizosphere of grasses, more than legumes are found to stimulate *Azospirillum* colonization (Dobereiner *et al*., 1976).

During external colonization, the bacteria often occur mainly in clusters, although many single cells may also be scattered on the root surface. These internally colonizing bacteria are embedded in the mucilage layer of the root surface (Bashan *et al*., 1987; Schank *et al*., 1979; Umali Gracia *et al*., 1981).

Azospirillum cells can colonize internally by penetrating into the root intercellular spaces (Patriquin and Dobereiner, 1978); Umali Gracia *et al*., 1981).

Bashan and Holgunin (1995) reported that colonization of the root system in soil was an active process as determined by bacterial motility. The *Azospirillum brasilense* wild type strain (mot+) was able to colonize to the roots of all the sixty four plant species tested. *Azospirillum* sp. were not found to be plant specific bacteria.

2.2 TAXONOMIC CONSIDERATION OF *AZOSPIRILLUM*

Beijerinck in 1922 first isolated the bacterium from N-poor sandy soil of the Netherlands and originally named as *Spirillum lipoferum* (Beijerinck, 1925). The bacterium was able to fix atmospheric nitrogen in enrichment culture. The importance of this microorganism was realized when Dobereiner and Day (1976) isolated the bacterium from the

roots of *Digitaria decumbens* cv. *Transvala* which exhibited nitrogenase activity. In this host, bacteria are in close proximity to the vascular tissue of the plant (endophytic) and have easy access to the photosynthates. The fixed nitrogen inturn is supplied directly to the plant. They also reported that *Spirillum lipoferum* as a very common root and soil inhabitant in the tropics. Subsequent taxonomic studies led to the creation of a new genus *Azospirillum* (Tarrand *et al*., 1978).

Azospirillum is gram negative, vibroid and 1 – 1.5 µm in diameter, possessing peritrichous flagella for swarming and a polar flagellum for swimming. It contain poly β-hydroxy butyrate (PHB) granules (Okon *et al*., 1976). Dobereiner *et al.* (1976) reported that *Azospirillum* is essentially an aerobic microorganism but able to grow under microaerophilic condition and only under the microaerophilic condition it is capable of fixing the nitrogen and not otherwise.

Based on DNA homology studies, Tarrand *et al.* (1978) included *Spirillum* under the new genus *Azospirillum* with two species *A. brasilence* and *A. lipoferum*. Another species, *A. amazonense* was isolated from roots of sugarcane and forage grasses (Magalhaes *et al*., 1983). It is more acid tolerant and was also found in roots of palm trees in the Amazon region. Reinhold *et al.* (1987) isolated and described *A. halopraeferans* from roots of Kallar grasses grown on salt affected soil in Pakistan. This species showed some adaptation to salinity and possessed remarkable growth and N_2-fixation at 40°C with 0.25 per cent salt in a medium. *Azospirillum irakense* with a lower G + C content and with ability to hydrolyse pectin was isolated by Khammas *et al.* (1989).

2.3 OSMOTOLERANCE

Among the different species of *Azospirillum* tolerance to high concentration of sodium chloride sucrose or polyethylene glycol increased in the order of *A. amazonense*, *A. lipoferum, A. brasilense* and *A. halopraferens* (Hartmann *et al*., 1991). In *A. irakense* growth occurred in the presence of one to three per cent NaCl (Khammas *et al*., 1989).

Nitrogenase activity was more sensitive to salt stress then cell growth on combined nitrogen (Rao and Venkateshwaralu, 1982). Under osmotic stress, growth of *A. brasilense* and *A. halopraferens* was stimulated by glutamate or proline, whereas no effect was found with *A. amazonense* and *A. lipoferum* (Hartmann and Burries, 1987). The latter two species efficiently used glutamate and proline as carbon source (Hartmann and Burries, 1988). Therefore *A. amazonense* and *A. lipoferum* may not be able to utilize aminoacids as compatible solutes during osmotic stress. Even low concentration of glycine and betaine were sufficient to improve osmotolerance. While *A. lipoferum* could not use them as osmoprotectants (Hartmann and Burries, 1988).

2.4 INTRINSIC ANTIBIOTIC RESISTANCE (IAR)

The genetic diversity of soil bacterium has been demonstrated using the patterns of intrinsic antibiotic resistance (Somesegaran and Hoben, 1994). They have been used extensively to mark the strains in genetics in and molecular biology experiments (Krishnaraj, 1996). The IAR is due to the presence of genes which are responsible for synthesis of enzyme systems present both on the main chromosome and plasmid (Hayes and Wolf, 1990) that detoxify the antibiotics and proteins that inhibit the cellular transfer of the antibiotics.

Within the species of *Azospirillum* difference have been found between strains isolated from comparable habitats or even from the same habitat (Balandreau, 1986). Dobereiner and Baldani (1979) found diversity among *Azospirillum* strain, with respect to their resistance to streptomycin, tetracycline, gentamycin and erythromycin.

Franche and Elmerich (1981) found that the wild type strains of *A. lipoferum* and *A. brasilense* were sensitive to streptomycin, rifampicin, naldixic acid, cephalovictin and cefalatin. While Singh and Wenzel (1982) reported that all the strains of *A. lipoferum* and *A. brasilense* were resistant to trimethoprim except *A. lipoferum* A23 which was highly sensitive.

Magalhaes *et al.* (1983) observed that all the *A. amazonense* strain were resistant to penicillin, but relatively tolerant to chloramphenicol and erythromycin. About 11 per cent of the total *Azospirillum* isolates showed the resistance to 50 ppm streptomycin and only 5 per cent of the total *Azospirillum* isolates recorded resistance to 50 ppm chloramphenicol (Govindan, 1993).

2.5 NITROGEN FIXATION BY *AZOSPIRILLUM*

Azospirillum strains fix atmospheric nitrogen efficiently as free living bacteria are associated with plant roots and participate in the nitrogen cycle (Henlin *et al*., 1987). Nitrogen fixation was the first major mechanism for the enhancement of plant growth by *Azospirillum*. Incorporation of atmospheric nitrogen into the host plant by *Azospirillum* was estimated mainly by the indirect acetylene reduction assay (Van Bevkum and Bohlool, 1980). Nitrogen fixation by *Azospirillum* has now been confirmed by several workers using not only conventional microkjeldal assay, but also by the more definite method of isotopic enrichment involving ^{15}N (Laxmikumari *et al*., 1976; Okon *et al*., 1976; Barbar *et al*., 1978).

In the past decade, there have been further reports of substantial increases in total N accumulated in the field grown crops following inoculation with *Azospirillum* (Bashan and Levanoy, 1990). In most experiments, increases have been less than 20 per cent (Bouton *et al*., 1979; Albrect *et al*., 1981; Reyndus and Vlassak, 1982). However, many reports also showed increases of 20-60 per cent (Bouton *et al*., 1979; Tyler *et al*., 1979; Kapulnik *et al*., 1981; Okon *et al*., 1981) and highly extreme values 60-128 per cent (Hegazi *et al*., 1981; Kapulnik *et al*., 1981; Subbarao, 1981; Baldani *et al*., 1983) in N uptake than uninoculated control.

Dobereiner and Boddey (1980) reported that the efficiency of nitrogen fixation increased with increasing age of culture, reaching values of 98 mg N per g lactate and 49 mg N per g of glucose for *A. brasilense* and *A. lipoferum* respectively in the early stationary phase. The nitrogen fixing potentials of local isolate of *Azospirillum* sp. varied between 16.2 and 23.96 mg per g of energy source (Purushothaman *et al*., 1988).

Prathibha *et al.* (1993) observed 2.4 to 18.23 mg N g^{-1} of carbon source utilized by *Azospirillum* strains from cotton genotypes cultivated in northern parts of Karnataka. Similarly, Maheshkumar (1997) isolated six *Azospirillum* strains from the root of bamboo plants and found them to fix 15.6 to 22.4 mg g^{-1} carbon source utilized.

Several workers have examined the nitrogen fixation efficiency of *Azospirillum* to fix as much as 150 g of N per gram of malate utilized. Okon *et al.* (1977) reported 20-24 mg N fixed per gram of carbon sources. Dobereiner and Boddy (1980) reported the efficiency of nitrogen fixation increased with increasing age of culture reaching values of 998 mg per gram carbon sources and 49 mg of glucose for *A. brasilense* and *A. lipoferum* respectively in the early stationary phase, Purushothaman *et al.* (1988) reported that the nitrogen fixation potential of *Azospirillum* sp. varied between 1.6 to 23.96 mg per gram of carbon by *Azospirillum* strains from cotton genotypes. Maheshkumar (1997) isolated six *Azospirillum* strains from the roots of bamboo plants and found them to fix 15.6 to 22.4 mg per gram of carbon source utilized. *A. lipoferum* and *A. brasilense* showed nitrogen fixation in the range of 7.54 to 24.53 mg of nitrogen per gram of malic acid after seven days at 28°C under static condition (Tamilvendan and Purushothaman, 1996). They reported that *Azospirillum lipoferum* and *A. brasilense* showed nitrogen fixation in the range of 7.54 to 24.53 mg of nitrogen g^{-1} of malic acid after seven days at 28°C under static conditions of the 88 *Azospirillum* isolates, 55 per cent were identified as *A. lipoferum* and 41.57 per cent as *A. brasilense*. These represented isolates from the rhizosphere / endorhizosphere of different ornamental plants. The nitrogen fixing capacity of these isolates ranged from 1.4 to 20.54 mg N g^{-1} of malate.

2.5.1 Hormones

Many *Azospirillum* strains produce plant hormones both in liquid culture and natural situation. The major hormone produced is indole-3-acetic acid (IAA) (Barbieri *et al*., 1986;

Fallik *et al*., 1989; Hartmann *et al*., 1983; Jain and Patriquin, 1985; Kolb and Martin, 1985; Tien *et al*., 1979). Other hormones detected at much lower but biologically significant levels were indole lactic acid (Tien *et al*., 1979), Indole-3-butyric acid (IBA) (Fallik *et al*., 1989), Indole-3-ethanol, indole-3-methanol (Crozier *et al*., 1988), unidentified indole compounds (Bashan and Levonany, 1990), several gibberellins (Bottini *et al*., 1989; Tien *et al*., 1979), abscisic acid (ABA) (Kolb and Martin, 1985) and cytokinins (Horemans *et al*., 1986; Tien *et al*., 1979).

Azospirillum converts tryptophan to indole acetic acid (Reynders and Vlassak, 1979). IAA, gibberellins and cytokinins were excreted by *Azospirillum brasilense* (Tien *et al*., 1979). They stated that root exudates of plants contained tryptophan which probably acted as precursor for the synthesis of IAA source.

While examining the plant growth promoting influence of *Azospirillum*. Michiels *et al.* (1989) and Sumner (1990) found that the colonization of root by *Azospirillum* resulted in more developed root system (Patriquin *et al*., 1983; Jain and Patriquin, 1984; Kapulnik *et al*., 1985). Gaskin and Hubbell (1979) attributed the plant growth response to inoculation with *Azospirillum,* to the production plant growth promoting substances. Tien *et al.* (1979) proposed that changes in the root morphology were caused by plant growth factors produced by *Azospirillum*. They found that inoculation of pear millet greatly increased proliferation of lateral roots and root hairs. Zimmer and Bothe (1988) have an alternative explanation for the plant growth promotion by *Azospirillum*. Their opinion was that the nitrate is formed due to nitrate respiration which will react with a substance in the cell and the product formed (could be ascorbate) which function as an auxin. Hence, they postulated that IAA and nitrate probably are the only factors causing an enhancement of the growth of roots of grasses.

Hartmann *et al.* (1988) noticed that the IAA producing capacity of *Azospirillum brasilense* was more than that of *Azospirillum lipoferum.* However, no gibberellins were produced by both the species. *Azospirillum* species possess more than one IAA biosynthetic pathway (Abdel-Salam and Kingmuller, 1987). Zimmer *et al.* (1991) isolated an *Azospirillum brasilense* gene which was responsible for the IAA production by *A. irakense*.

The *Azospirillum* cultures containing 10^8 cfu per ml produced 32-40 mg of IAA per ml when grown on tryptophan free liquid medium (Fallik *et al*., 1989). The indole acetic acid produced from bacterial cell was considerably higher in the presence of ammonia than in N free medium. Tryptophan stimulated the IAA production and the concentration of IAA was low during logarithmic growth phase and increased rapidly with beginning of stationary phase (Omay *et al*., 1993). *Azospirillum* sp. Produced IAA in the late stationary phase and showed a significant increase in the IAA due to the presence of tryptophan (Baca *et al*., 1994).

An *Azospirillum* strain and a mutant, which over produced IAA in culture strongly affected plant root morphology (Jain and Patriquin, 1985; Kolb and Martin, 1985). However, mutants that failed to produce IAA in culture had no effect on root morphology (Barbieri *et al*., 1986 and 1990). Inoculation with *Azospirillum* improved the hormonal balance of a hormonal defective mutant of wheat (Inbal and Feldman, 1992). The phytohormone synthesized by *Azospirillum* influenced the root hair development, respiration rate, metabolism and root proliferation which inturn resulted in better mineral uptake of the inoculated plant (Bar and Okon, 1993).

2.5.2 *Azospirillum* – Maize association

Paredes *et al.* (1988) reported that in pot trials when maize seedlings were inoculated with *Azospirillum brasilense* root dry matter content increased by 22 to 118 per cent and total N content increased 101 per cent. The strains which produced more IAA were more effective in enhancing plant growth.

Arsae *et al.* (1990) observed increase in epicotyl and hypocotyls length at 5 DAG (Days after germination), root and shoot length at 14 DAG due to seed inoculation with *A. lipoferum*.

Berge *et al.* (1990) reported that soil inoculation of *A. lipoferum* supplied with 160 kg N/ha, gave an increased grain yield (14.41 t/ha) compared to uninoculaed control (12.26 t/ha).

Morandini *et al.* (1990) reported that, during root colonization the number of microorganisms found inside the maize root may be comparable regardless of the genotype, which have different growth resources.

Percira (1990) reported that inoculation of *A. amazonense* and *A. lipoferum* increased grain yield of maize by 20 and 60 per cent respectively.

Zaady and Okon (1990) reported treatment of malate grown cells with 20 mM D-fructose for 7.5 min, or with maize root extracts, significantly promoted the subsequent adsorption of *Azospirillum* cells to maize roots.

Stancheva *et al.* (1991) observed an increased total N and protein N content, dry weight and nitrate reductase activity when seeds were inoculated with *Azospirillum brasilense* then from uninoculated control, especially at low N rates.

The interaction between *Azospirillum brasilense* and the maize root system increased the plant biomass and total N content (Stancheva and Dinev, 1992). Inoculated plants had a higher dry matter accumulation after milk ripeness than non-inocualted plants. The influence of inoculation on DM accumulation being greatest with 100 kg N/ha. DM yield with this combination were equivalent to those with 200 kg N/ha alone and also observed increased grain yield and nitrate reductase activity (Stancheva *et al*., 1992).

In field trials, application of peat based inoculant of *A. brasilense* as well as a granular inoculant in the seed furrows of maize resulted in significantly increased yields (11 to 14%) at low rates of N application (Fallik and Okon, 1996).

Hernandez *et al*. (1997) conducted pot culture experiment, maize inoculated with *Azospirillum* gave similar shoot dry weight to the application of 100 kg N per ha.

Ribando *et al.* (1998) reported that, inoculation with *Azospirillum* showed a significant increase in the mean dry weight of shoot and root at the milk ripeness sage. In general, inoculated plants showed higher NR activity in both leaves and roots.

Casanovas *et al.* (2000) reported that growth promoting effect of *Azospirillum* on root dry matter and radical surface of maize seedlings was found to be significant upon seed inoculation with *Azospirillum.* Maize productivity was increased by 17 per cent (from 5211 to 6067 kg/ha), while maize cob length increased from 13.6 to 14.4 cm (Cavallet *et al*., 2000).

Woodard and Bly (2000) noticed, increased shoot dry matter production as well as grain yield upon inoculation of maize seeds with N2 fixing bacteria, *Azospirillum brasilense*.

Swedrzynska and Sawicka (2001) reported that inoculation of cereals with *A. brasilense* bacteria lead to increase their number in soil and also application of mineral nitrogen to the crop was favourable for the multiplication of *Azospirillum*.

Devi (2002) reported that forage yield of maize is influenced by nitrogen level and biofertilizer. Where the fodder maize variety “African tall” produced significantly grain forage and dry matter yield at higher dose of N plus biofertilizer. Casanavas *et al.* (2003) reported that *Azospirillum* inoculation will improve the physiological status of maize plant which could account for the amelioration of the harmful effects of water short fall during the flowering period which will help in increase in grain production.

Creus *et al.* (2004) reported that grain yield loss due to drought in non-inoculated plant was 26.5 per cent compared with inoculated plant (14.1%) and also grain harvested from *Azospirillum* inoculated plant has significantly higher Fe, Mg, K and Co than non-inoculated plants. A pot experiment to evaluate the possible roles of nitrogen fixation and/or enhanced mineral uptake by *Azospirillum lipoferum* and *Bacillus polymyxa*, inoculation in

improving salt tolerance of maize plant and they reported that plant inoculated with N2 fixers resulted in an increase in fresh and dry matter as well as water content of plant.

2.6 IMPROVEMENT OF BENEFICIAL TRAITS OF *Azospirillum* BY MUTATIONS

De-Gallo *et al.* (1982) reported that ethyl methyl sulfonate (EMS) and nitrosoguanidine (NTG) were more effective in obtaining mutation frequency in the *Azospirillum brasilense*. Significant improvement in the symbiotic nitrogen fixation was observed using azide resistant mutants *Rhizobium leguminosarum* (Ram *et al*., 1978).

Rai (1985) isolated NTG treated, five antibiotic resistant mutant strains. Mutant strain STR 112 and KR 2051 showed maximum nitrogenase activity glutamine synthetase activity and hydrogenase activity. Inoculation of Bengalgram genotypes with *Azospirillum brasilense* and its mutants led to significant increase in associative nitrogen fixation, dry weight of roots and grain yield compared with the unionculated control.

Machado *et al.* (1990) isolated spontaneous ethylenediamine resistant mutants of *Azospirillum brasilense* and noticed that they possessed higher excretion of NH4 than wild type. The nitrogenase activities of these mutants on nitrogen free minimal medium were higher than that of wild type. These NH^{+}_{4} excreting mutants enhanced the nitrogen supply to wheat. The ammonium excreting *Azospirillum* sp. became intercellularly established in maize paranodules (Weniger and Vanderleyden, 1994) and the ammonium excreting *Azospirillum* could establish itself in the induced tumours along stem and roots of rice (Weniger, 1997).

Mutants of *Azospirillum brasilense* altered in the uptake of iron (Mori *et al*., 1992) excreting high amounts of IAA (Hartmann *et al*., 1983) and altered nitrogenase activity were isolated (Rai, 1985).

Tamilvendan and Purushothaman (1996) mutagenized the *Azospirillum* strain using the sodium azide. Mutants were expressed relatively with higher phosphate solubilizing activity than the standard. The mutant also retained higher nitrogenase activity and nitrogen fixing ability.

Van-Domellin *etal* . (1997) genetically characterized spontaneous EDA (ethylenediamine) resistant mutants of *A. brasilense* and reported that *A. brasilense* ammonia excreting mutants seem to be defective in their ammonium assimilatory enzymes. Hence the activity and/or synthesis of nitrogenase enzyme in diazotrophs are not inhibited by an abundant of fixed nitrogen in the environment. Thus, even in plentiful supplies of ammonia, nitrogenase activity may not be affected.

Gerk *et al.* (2000) reported that the mutation affecting the flocculation, differentiation into cyst like forms and the root colonization enhanced the nitrogenase activity in hydroponics *Azospirillum brasilense* strain Sp245 and Sp7 in association with wheat plant. Chemically induced *Azospirillum brasilense* glutamine synthase mutant offer the possibility to generate ammonium excreting *Azospirillum* strains.

Dayamani (2003) reported sodium azide resistant strains ACD-802 and ACD-701 of *Azospirillum* that fixed more amount of nitrogen 63.01 and 58.8 mg g^{-1} of malate compared to their wild types ACD-8 and ACD-7 that fixes 18.83 and 18.40 mg g^{-1} of malate respectively.

2.7 POPULATION DYNAMICS STUDIES

Weniger (1992) studied on the marked *Azospirillum brasilense* by insertion of transposon Tn5 into its genome. The Tn5 insertion did not interfer with physiological characteristics as nitrogen fixation, auxin production and nitrogen reduction nor with the growth rate of bacterium. He further reported that the detection limit of the technique was as low as approx 25 cells per gram dry soil.

Belimov *et al.* (1995) conducted pot and filed experiments to study the population dynamics of associative nitrogen fixers viz., *Azospirillum lipoferum* 137, *Arthrobacter mysorens* 7, *Flavobacterium* sp. 130 and phosphate-solubilizing strain of *Agrobacterium radiobacter* in soil and the rhizoplane of inoculated plants and he reported that no correlation was established between survival of introduced bacteria and their effect on the plant development. Further it was concluded that the influence of plant on survival of bacteria was not specific. In contrast, the plant response to inoculation was conditioned to a greater extent by the plant genotype.

Alexandre *et al.* (1996) isolated *A. lipoferum* 4B and non-motile *A. lipoferum* 4T from rice to study the population dynamics of motile and non-motile *A. lipoferum* strain during rice root colonization in rhizosphere. Inoculation experiments showed the *A. lipoferum* 4T, but not *A. lipoferum* 4B, needed rice root to stabilize in sterile soil. Both strains were able to colonize efficiently rice roots (10^8 cfu g^{-1} fresh roots) but motile form 4B remained dominant. In spite of their phenotypical differences. *A. lipoferum* 4B and 4T coexisted without exclusion in sterile soil (planted or not) and rice rhizosphere.

2.8 COMPETITIVENESS STUDIES

Symbiotic effectivity and competitiveness of Azide Resistant mutants of *Rhizobium* sp. (Vigna) was studied by Yadav *et al.* (1992). He derived azide resistant (Azi^r) mutants from two cowpea Rhizobium strains S24 and CT2014 (Vigna group) by γ-Irradiation which tolerated upto 25 mg ml^{-1} of sodium azide. Eleven Azi^r mutans of strain S24 and 12 Az^r mutants of CT2014 were randomly selected and tested for symbiotic effectivity with green gram (*Vigna radiata*) as a test host. Azide resistant mutants with both increased and decreased symbiotic effectivity were observed. However, 3 mutants of S24 and 2 mutants of CT2014 showed increase in effectivity under sterilized chillum jar conditions. Azide resistance in the effective mutant strains was associated with the increased tolerance of nitrogenase to ammonium ion under *ex planta* conditions. Two of the Azide resistant mutants of strain S24 were also superior in symbiotic effectivity under unsterilized conditions and formed 87-96 per cent nodules in competition with native rhizobia. Introduction of azide resistance in this group of rhizobia therefore, could be taken as a parameter for strain competitiveness in soil.

A few research groups have examined the effect on competition of marking rhizobia with the intact transposon Tn5. Sharma *et al.* (1991) found that Tn5 insection did not affect the competitive ability of two strain of chickpea rhizobia which had wild type fixation abilities. Brockman *et al.* (1991) studied the symbiotic effectiveness and competitive ability of *R. leguminosarum S. V. Viciae* and *R. leguminous* bv. phaseoli strains marked variously with spontaneous antibiotic resistance or transposon Tn5 in sections, the Tn5 containing strains exhibited apparently increased symbiotic effectiveness.

Lam *et al.* (1990) studied the genetic approaches for studying rhizosphere colonization. In one of his study a Tn5 derivative containing a constitutively expressed β-galactosidase (lac Z) gene was used to generate a collection of insection mutants which could be distinguished from the wild type parent on xgal plates. Each mutant was examined for its ability to colonize wheat seedlings in the presence of the wild type parent and found that the ability of these mutants to compete varied over a wild range.

Sessitsch *et al.* (1996) measured the competitiveness index of *Rhizobium tropici* strain CIAT 899 derivatives marked with the gus-A gene and showed that competitiveness indices of the four gus-A marked strains varied etween the experiment, but in each case, they appeared either equally competitive or more competitive than the parent strains.

III. MATERIAL AND METHODS

Experiments were conduced to study the effect of Azide resistant mutants of *Azospirillum* on nutrient uptake, growth and yield of maize (DMH-2) in the Department of Agricultural Microbiology and Department of Plant Biotechnology, University of Agricultural Sciences, Dharwad, during the year 2004 and 2005. The details of the material used and the techniques employed during the course of investigation are presented in this chapter.

3.1 *Azospirillum* STRAINS

The following five strains of *Azospirillum* were obtained from the culture collections of the Department of Agricultural Microbiology and used for the present study.

a. *Azospirillum* ACD-15

b. *Azospirillum* ACD-802

c. *Azospirillum* ACD-8

d. *Azospirillum* ACD-701

e. *Azospirillum* ACD-7

These strains were identified and maintained on nutrient agar and stored at 4°C.

3.2 SODIUM AZIDE STOCK

The sodium azide stock was prepared by using the chemical from Hi-media, 1000 ppm stock was prepared and sterilized separately and added to the medium just before pouring the different concentration used in the experiment viz., 5, 10, 15, 20, 30, 35 and 40 ppm. The azide was supplemented to the Luria Agar and N-free malate agar.

3.3 IDENTIFICATION OF THE ISOLATES

The five identified *Azospirillum* strains viz., ACD-7, ACD-8, ACD-802, ACD-701 and ACD-15 were subjected to morphological and physiological characterization as given below.

3.3.1 Morphological characterization

3.3.1.1 Colony morphology

The *Azospirillum* form characteristic colonies on different media which could be a tool for preliminary identification.

Each isolate was streaked on two petriplates containing Nfb malate agar and incubated for seven days. Typical small white translucent colonies were observed. Similarly the isolates were also streaked on the plates containing potato BMS agar and congo red medium. Typical pink coloured colonies on potato BMS agar (Baldani and Dobereiner, 1980) and scarlet colonies (Rodriguez-Cacaves, 1982) were taken as preliminary indication that the isolates were *Azospirillum*.

3.3.2 Microscopic observation

The bacteria isolated from maize crop of different locations were studied for cell morphology, Gram reaction and motility. The Gram staining was done using 24-hour-old culture by the procedure of Huckers modification (Rangaswami and Bagyaraj, 1996). The

stained cells were observed under a microscope with oil immersion lens. The Gram reactions and cell morphology were recorded.

The motility of the bacterial isolates was tested by keeping a drop of 24 hours old inoculum grown in nutrient broth into cavity slide (hanging drop technique). Isolates with typical spiral (Cork-Screw) motility was taken to be *Azospirillum*.

3.3.3 Physiological tests

The physiological studies viz., acid from glucose, catalase activity and urease test were carried out for identification of *Azospirillum*.

3.3.3.1 Acid from glucose (Tarrand *et al*., 1978)

A five ml of glucose peptone broth with Bromothymol Blue (BTB) was dispensed into test tubes and autoclaved at 15 lbs for 20 minutes. Overnight cultures were suspended in sterile phosphate buffer saline and were inoculated (0.1 ml) into the medium. The tubes were incubated at 37°C for three days. Color change in BTB from green to yellow indicated acid production.

3.3.3.2 Catalase activity

To test the isolates for the presence of catalase enzyme, 3 per cent hydrogen peroxide was added to the single colony on Nfb medium and observed for fizzling to confirm its presence.

3.3.3.3 Urease test

Filtered and sterilized urea broth concentrate was aseptically added to the sterilized and cooled distilled water; 3 ml of this solution was dispensed into the sterile tubes aseptically.

Urea broth + phenol red + culture

Pink colour → + for the test

No pink colour – for the test.

3.3.3.4 Oxidase test

Three drops of tetramethyl-P-phenylenediamine dihydrochloride regent was transferred on to the surface of the growth of each test organism. A colour change from pink to maroon and finally to black indicates positive for the test.

3.3.3.5 *In vitro* nitrogen fixation

Nitrogen fixation by each *Azospirillum* strain was studied according to the method described by Humphries (1956).

The N free semi solid malate medium supplied with L-glutamic acid @ 100 mg/L was used in this study. To a 250 ml conical flask 100 ml of the above medium was dispensed and autoclaved at 15 lbs pressure for 15 minutes. The *Azospirillum* isolates were grown separately for 24 hrs in Nfb broth and inoculated @ 2 ml/100 ml of the medium. Duplicate samples were kept for each isolate. The flask were incubated at 37°C for seven days.

After seven days of incubation the culture was homogenized. Five ml of the homogenized culture was withdrawn and digested with 5 ml concentration H_2SO_4 and 200 mg catalytic mixture (K_2SO_4 : $CuSO_4$, selenium) (100:10:1 ratio) until the contents become clear. After cooling, the volume was made up to 25 ml with distilled water. Then 5 ml of aliquot was

transferred to microkjeldhal distill unit. An aliquot of 10 ml of 40 per cent sodium hydroxide was added and steam distilled. Ammonia evolved was collected over 2 per cent boric acid (20 ml) containing 2 drops of double indicator (83.3 mg bromocresol green + 16.6 mg methyl red indicator dissolved in 10 ml of 95% ethanol) and back titrated against 0.05 N H_2SO_4. Using the titre value and the formula of one ml of 0.50 N of H_2SO_4 = 0.0007 g.

3.4 ESTIMATION OF GROWTH PROMOTING SUBSTANCES

3.4.1 IAA

Indole acetic acid (IAA) production by *Azospirillum* isolates was estimated by the method descried by Tien *et al.* (1979).

Hundred ml of N-free malate broth containing 0.005 M L-tryptophan was sterilized in 250 ml conical flask. Each isolate of *Azospirillum* was grown overnight in nutrient broth was inoculated to the above medium and incubated at 37°C for seven days in dark. Duplicate flasks were maintained for each isolate. The cultures were centrifuged at 6000 rpm at refrigerated centrifuge at 4°C and the supernatant was collected in a conical flask and used for estimation of IAA.

Twenty-five ml of supernatant was taken in a 100 ml conical flask and pH was adjusted to 2.8 using 1N HCl. Equal volume of diethyl ether was added to it and incubated in dark for four hours. Diethyl ether extractions of IAA was done in a separating funnel. The organic phase was discarded and the solvent phase was pooled and evaporated to dryness. To the dried material, two ml of methanol was added and the IAA present in the methanol extract was determined using the method explained by Gorden and Paleg (1957). To 0.5 ml of methanol extract, 3 ml of distilled water and 4.0 ml of Saplor's reagent (1.0 ml of 0.5 M FeCl3 in 50 ml of 35 per cent perchloric acid) was added and incubated in dark for one hour. The intensity of pink colour developed was read at 535 nm in a spectrophotometer. From a standard curve prepared with known concentrations of IAA, the quantity of IAA in the culture filtrate was determined and expressed as µg per 100 ml of the medium.

3.4.2 Gibberellic acid

The gibberellic acid produced by the *Azospirillum* isolates was estimated by following the method described by Paleg (1965). *Azospirillum* isolates were inoculated to sterilized nitrogen free semi solid malate medium in duplicates. Seven days after incubation, twenty-five ml of the culture filtrate was taken in a test tube to which two ml of zinc acetate was added. After two minutes, two ml of potassium ferrocyanide was added and centrifuged at 1000 rpm for 15 minutes. To five ml of this supernatant, five ml 30 per cent HCl was added and incubated at 20°C for 75 minutes. The blank sample was treated with five per cent HCl. The absorbance of the samples as well as blank was measured at 254 nm in UV spectrophotometer. The amount of GA present in the extract was calculated from the standard curve and expressed as µg/25 ml of the medium. The standard curve for GA was prepared by using graded concentrations of GA.

3.5 INTRINSIC ANTIBIOTIC RESISTANCE (IAR) OF STRAINS

To mark the strains chosen for further detailed analysis, the IAR of two wild types ACD-7 and ACD-8 and two mutants ACD-802 and ACD-701 and a reference strain ACD-15 was examined. Plates of nutrient agar containing different concentration of antibiotic were prepared 10 µl of the overnight culture was spotted on the plates and incubated at 30°C for 12 hrs. Growth was assessed qualitatively by comparing with the growth on control plates. The antibiotics used their source and concentration are given in the Table 1.

3.6 RESISTANCE OF THE *Azospirillum* ISOLATES TO SODIUM AZIDE

Sodium azide resistance of isolates was tested at different concentrations viz., 5, 10, 15, 20, 25, 30, 35 and 40 ppm of sodium azide on N free malate solid media. The medium and sodium azide solutions were separately sterilized and mixed just before pouring the medium on to the sterile plates according to the concentration required. On setting of the agar, 10 µl of the inoculum from each *Azospirillum* isolates were spotted. The plates were then incubated at 37°C for four days. Growth on the sodium azide was recorded and compared with control.

3.6.1 Preparation of malate and Luria medium

The N free bromothymol blue broth supplied with one g of yeast extract was used for the present study. TO a 250 ml conical flask 100 ml of the above medium was dispensed and sterilized in an autoclave at 121°C for 15 min. Similarly 100 ml of 1000 ppm stock solution of the sodium azide was prepared separately and sterilized in an autoclave at 121°C for 15 min, after sterilization broth was supplied with different concentration of azide viz., 5, 10, 15, 20, 25, 30, 35 and 40 ppm. Similarly, Luria Agar was also supplemented with above concentration of azide from the stock prepared.

3.6.2 Inoculum preparation and inoculation

The *Azospirillum* strains were grown in modified Okon's medium for four days incubated on shaker at ambient temperature (28 ± 2°C). After incubation, one ml of inoculum was inoculated to 100 ml of pre-sterilized Nfb malate broth supplemented with one g per litre yeast extract containing different concentration of sodium Azide and kept for incubation at room temperature (26-30°C) for four to six days.

The initial population count was taken using standard plate count method.

3.6.3 Population count

On 7^{th} day of inoculation, population count was taken on modified Okon's medium by standard plate count method. The plates were incubated at 30°C for two days and the population was observed and expressed as $\log_{10}$ cfu per ml of broth.

3.7 FIELD EXPERIMENT

Field experiment was conduced to study the influence of inoculation of azide resistant mutants on growth, yield and uptake of nitrogen by maize crop under rainfed conditions at Main Agricultural Research Station, Dharwad during 2004-05. The details of the materials used and the techniques adopted during the course of investigation are described below.

3.7.1 Location

The field (Plot No. 125 of E block of MARS, University of Agricultural Sciences, Dharwad) is situated in the transitional tract of Karnataka state at 15°26' North latitude, 75°71' East longitude and at an altitude of about 678 m above the mean sea level.

3.7.2 Soil characteristics of experimental site

The soil of the experimental site was medium black clay in nature. Composite soil sample upto a depth of 30 cm was collected from the experimental site, before initiating the experiment and was analysed for important physical and chemical properties as well as microbial population by employing standard methods. The results are presented in the Table 2.

Table 1. Antibiotics and their concentrations used in the study

Sl. No.	Antibiotic	Abbreviation	Solubility	Concentration (ppm)			Source
1.	Ampicillin	Amp	St. dist. H_2O	100	150	200	Ranbaxy, India
2.	Chloramphenicol	Clm	Alcohol	50	100	200	Sigma chemicals, USA
3.	Gentamycin	Gen	St. dist. H_2O	50	100	150	Ranbaxy, India
4.	Kanamycin	Kan	St. dist. H_2O	50	100	200	Hi Media, Mumbai
5.	Nalidixic acid	Nal	0.2 N KOH	10	20	25	Hi Media, Mumbai
6.	Spectinomycin	Spc	St. dist. H_2O	20	40	60	Sigma chemicals USA
7.	Hygromycin	Hyg	St. dist. H_2O	50	100	150	Sigma chemicals USA

Table 2. Physical and chemical properties of the soil of the experimental site

	Particulars	Value obtained	Method employed
I.	Physical properties		
a.	Mechanical composition		
	Coarse sand (%)	5.80	Hydrometer method (Piper, 1966)
	Fine sand (%)	14.20	Hydrometer method (Piper, 1966)
	Silt (%)	28.00	Hydrometer method (Piper, 1966)
	Clay (%)	51.90	Hydrometer method (Piper, 1966)
	Textural class	Clay loam	
b.	Bulk density (g cm^{-3})	1.20	Core sample method (Dastane, 1967)
II.	Chemical properties		
	Total nitrogen (%)	0.056	Subbiah and Asija (1956)
	Available phosphorus (kg/ha)	42.00	Olsen's method (Jackson, 1967)
	Available potassium (kg/ha)	325.00	Flame photometer (Jackson, 1967)
	Soil pH (1:2.5, soil : water)	7.50	pH meter (Piper, 1966)
	Organic carbon (%)	0.76	Wet oxidation method (Jackson, 1967)

Table 3. Monthly meteorological data for the experimental year (*kharif*, 2005) and the mean of past 54 years (1950-2004) as recorded at the Meteorological Observatory, Main Agricultural Research Station, University of Agricultural Sciences, Dharwad (Karnataka)

Month	Rainfall (mm)		Temperature (°C)				Relative humidity (%)	
			Mean maximum		Mean minimum			
	2005	Mean*	2005	Mean*	2005	Mean*	2005	Mean*
January	0	0.08	29.9	29.61	12.9	14.67	52	63
February	0	1.14	33.4	32.52	14.8	16.37	62	51
March	0	0.14	36.0	36.49	18.9	19.59	42	56
April	75.0	48.88	36.3	37.38	21.3	19.83	53	76
May	29.4	80.45	37.0	33.66	21.5	21.40	55	66
June	151.0	109.86	30.9	28.84	21.4	21.50	76	81
July	290.2	148.33	27.4	29.17	21.5	21.01	83	87
August	138.8	96.09	27.1	27.00	20.4	20.30	81	86
September	194.5	102.21	27.5	28.58	20.3	19.91	85	82
October	89.4	130.15	29.6	30.09	19.1	18.41	70	76
November	38.0	32.11	29.4	30.19	14.9	15.88	51	68
December	0.0	53.51	28.9	29.39	13.1	12.51	53	63
Total	1006.3	802.52						

*Mean of 54 years (1950-2004)

3.7.3 Agro-climatic conditions

The data on climatic parameters such as rainfall, maximum and minimum temperature and relative humidity recorded at meteorological observatory, Main Agricultural Research Station, Dharwad during experimental year 2004-05 is presented in Table 3.

3.7.4 Previous crop on the experiment site

During the previous *kharif* season, 2004, Green gram was grown on the experimental site.

3.8 DETAILS OF FIELD EXPERIMENT

Sl. No.	Particulars	Details
1.	Cultivar	DMH-2
2.	Design	Completely Randomized Block Design
3.	Treatments	T1 – ACD-15 + 75% RDN
		T2 - ACD-15 + 100% RDN
		T3 – ACD-802 + 75% RDN
		T4 - ACD-802 + 100% RDN
		T5 – ACD-8 + 75% RDN
		T6 - ACD-8 + 100% RDN
		T7 – ACD-701 + 75% RDN
		T8 - ACD-701 + 100% RDN
		T9 – ACD-7 + 75% RDN
		T10 - ACD-7 + 100% RDN
		T11 – UIC + 100% RDN
4.	Replication	Three
5.	Plot size	
	Gross plot	4.5 m x 4.5 m (20.25 m^2)
	Net plot	4.0 m X 4.2 m (16.8 m^2)
6.	Spacing	60 cm x 30 cm
7.	Fertilizer	100:50:25 NPK kg ha^{-1}

3.9 CULTURAL OPERATIONS

3.9.1 Land preparation and layout

The experimental site was brought to fine tilth by ploughing once with tractor iron plough and was followed by two harrowing. The experiment was laid out in RCBD as per the plan given in Fig. 1 and the plots were provided with bund all around to avoid contamination of soil and water from other plots. Irrigation channels were also made to facilitate irrigation.

3.9.2 Fertilizer application

A recommended fertilizer dose of 100:50:25 N:P:K kg ha^{-1} and FYM at 7.5 t ha^{-1} with nitrogen in two split as per treatment (50% nitrogen as urea was supplied as basal dose and remaining as top dressing at 40 days after sowing were applied). FYM and other major nutrients i.e., P and K were applied in the form of SSP and MOP respectively to each plot at the time of sowing in small furrows opened with a marker and mixed thoroughly with the soil.

3.9.3 Seeds and sowing

Certified seeds of DMH-2 hybrid maize were used for sowing at the rate of 15 kg ha^{-1}. For seed treatment, the *Azospirillum* mutants were grown in N free malate, broth which contain azide of different concentration (30 and 40 ppm for ACD-701 and ACD-802 respectively) and kept for incubation at room temperature (26-30℃) for four to six days. After the growth was attained, it was treated to seed. Before treating *Azospirillum* the seeds were coated with 5 per cent carboxy methyl acetate. And then 0.1 ml of inoculant was treated to 5 kg seed. After that coating was done with calcium carbonate. The seeds were dried in shade before sowing.

3.9.4 Irrigation

Immediately after sowing, plots were irrigated. The irrigations were scheduled at regular interval. Irrigation was stopped when the crop attained physiological maturity.

3.9.5 After Care

Intercultivation was carried out two times at 25 and 40 DAS. Hand weeding was done thrice in the experimental plot, soon after sowing, 30 DAS and 45 DAS to keep the plots free from weeds.

3.9.6 Harvesting and threshing

The crop was harvested when it attained full maturity (110 days after sowing). The cobs from the net plots were collected, air dried, threshed and seed yield per plot was recorded.

3.9.7 Collection of experimental data

For recording various biometric observations in the experiment, sample consisting of five plants were selected at random from each net plot. For each sample, observations on plant growth parameter and nitrogen uptake were recorded at different stages of the crop growth (30, 60 and 90 DAS). Yield parameters were recoded at harvest, whereas *Azospirillum* spp count in endorhizosphere were recorded at all stages of crop growth.

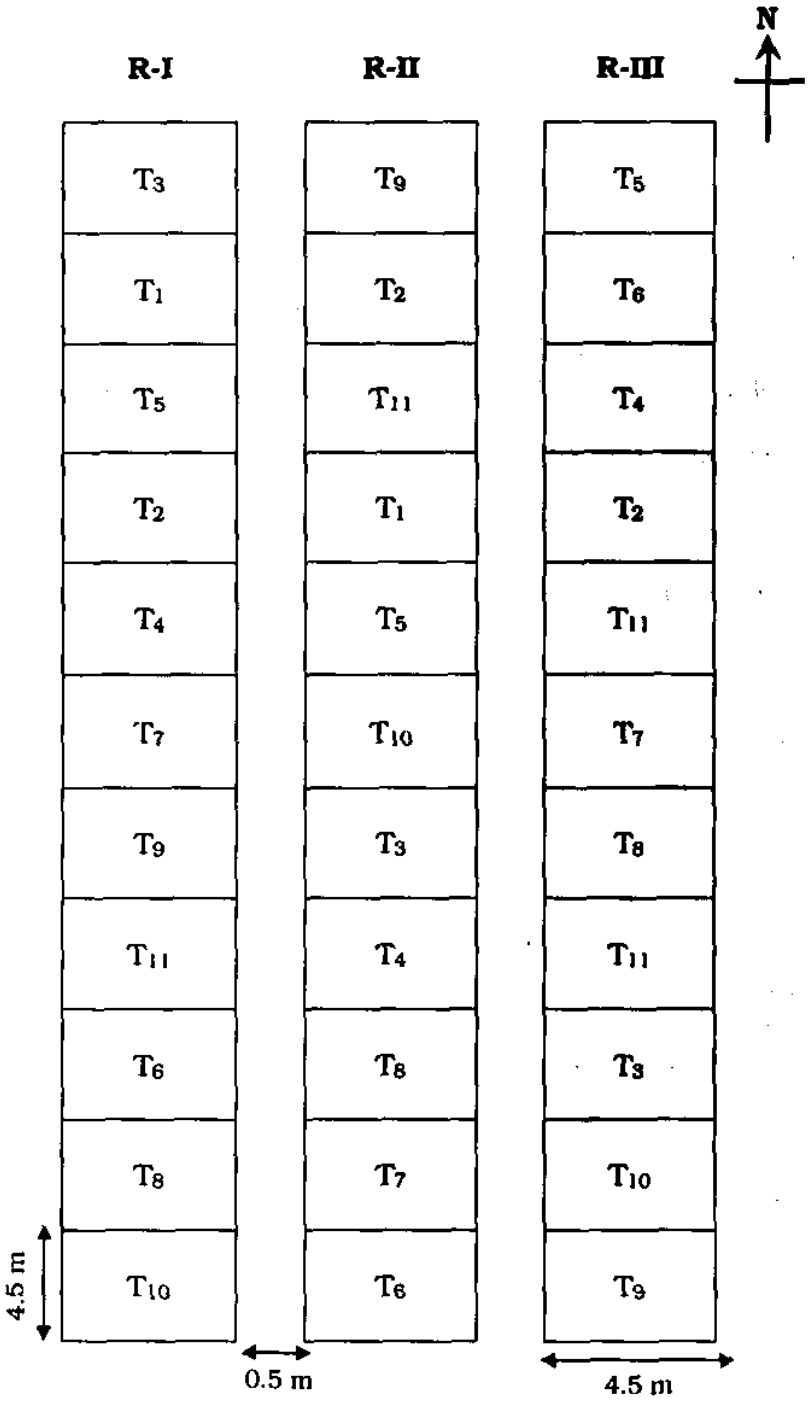

Figure 1: Plan of layout of the experiment

Figure 1: Plan of layout of the experiment

3.10 MICROBIOLOGICAL ASSAY

The roots were cut into 3-5 cm pieces and washed with sterile water six times and then macerated. These macerated tissues were used to enumerate *Azospirillum* sp. on semi solid NFb medium poured in tubes. The most probable number of *Azospirillum* sp was calculated from statistical tables of Cochran (1950) and expressed as MPN per gram of dry root.

3.11 PREPARATION OF PLANT SAMPLE

Plant sampling was done at 30, 60 and 90 DAS for nutrient uptake studies. After the harvest, the plants were washed immediately with tap water followed by 0.1N HCl and double distilled water. The plant samples were air dried for two days and then in a hot air oven at 65℃ to constant weight. The plant sample was then powdered in an electrical grinder and was preserved for further chemical analysis.

3.12 PLANT GROWTH PARAMETERS

3.12.1 Plant height

The height from the base of the plant to the fully opened leaf was recorded as plant height (cm) at 30, 60, 90 DAS and at harvest.

3.12.2 Number of leaves per plant

Numbers of fully opened green leaves per plant were counted for five plants taken for observation. The mean value was obtained for each treatment.

3.12.3 Dry matter accumulation

Harvested plants were dried under room temperature for two days and then dried in a hot air oven at 65℃ till get constant weight and weights were expressed as Gram per plant.

3.12.4 Stem girth

The diameter of the stem at the base of plant for the five plants was taken for the observation. The mean value was retained for each treatment.

3.13 CHEMICAL ANALYSIS

3.13.1 Chlorophyll content in leaf

Chlorophyll in leaf tissue was estimated through Arnon (1940) acetone method. The principle of estimation involves the adsorption of light by aqueous acetone extracts of chlorophyll at two wave lengths and setting up simultaneous equation using the specific absorption coefficients for chlorophyll ‘a’ and ‘b’.

Fresh leaves of 0.25 g was cut into small pieces and homogenized with pure acetone in mortar with pestle. The supernatant was decanted through a Whatman No. 42 paper into a 25 ml volumetric flask add 80 per cent acetone to the residue in the mortal and repeat the extraction until reside is decoloured. Then make upto the volume to 25 ml with 80 per cent acetone and measure the absorbance of the extract at 663 and 645 nm in spectrophotometer using 80 per cent acetone black.

Calculations

$$\text{Chlorophyll 'a'} = (12.7 \times A_{663}) - (2.69 \times A_{645}) \times \frac{V}{1000 \times a \times W} \text{ (mg/g)}$$

$$\text{Chlorophyll 'b'} = (22.9 \times A_{645}) - (4.68 \times A_{663}) \times \frac{V}{1000 \times a \times W} \text{ (mg/g fr. Wt).}$$

$$\text{Total chlorophyll} = 20.2 (A_{645}) - 8.02 (A_{663}) = X \frac{V}{1000 \times a \times W} \text{ (mg/g fr. Wt.)}$$

where,

A = Absorbance at specific wavelengths (645 and 663 nm)

V = Final volume of chlorophyll extract

W = Fresh weight of the sample (g)

a = Path length of light (1 cm)

3.14 YIELD PARAMETERS

3.14.1 Cob length (cm)

Five cobs were randomly selected from the samples collected from each plot measurement was done from base to up of the cob and expressed in centimeter (cm).

3.14.2 Cob weight (g plant^{-1})

Total cob weight obtained from five plants were recorded from each treatment mean was expressed as g per plant.

3.14.3 100-seed weight (g)

Weight of 100-seeds, randomly selected from net plot yield was recorded as 100-seed weight.

3.14.4 Seed yield (q ha^{-1})

Computation of seed yield was done in quintal per hectare (q ha^{-1}) from seed yield obtained from each net plot.

3.15 NUTRIENT UPTAKE

3.15.1 Nitrogen content (%) and uptake (mg/plant)

Nitrogen content (%) of shoot and root was estimated by modified micro kjeldhal method (Jackson, 1967) at 30, 60 and 90 DAS.Take 0.5 g of dried shoot and root sample which is in powdered form and digested with 5 ml of concentrated H_2SO_4 and 200 mg digestion calalyst (K_2SO_4:$CuSO_4$:Selenium) (100:10:1 ratio) until the contents become clear. After cooling, the volume was made upto 25 ml with distilled water. Then 5 ml of aliquot was transferred to micro kjeldhal distillation unit. An aliquot of 10 ml of 40 per cent sodium hydroxide was added and steam distilled. Ammonia evolved was collected over 2 per cent boric acid (20 ml) containing 2 drops of double indicator (83.3 mg bromocresol green 16.6 mg methyl red indicator dissolved in 10 ml of 95 per cent ethanol) and back titrated against 0.05

N H_2SO_4. Nitrogen uptake was expressed as percentage at different growth stages. The total N uptake was calculated for each treatment separately using the following formula,

$$\text{Nutrient uptake (g/plant)} = \frac{\text{Per cent nutrient concentration (N)} \times \text{biomass (g/plant)}}{100}$$

3.16 STABILITY AND COMPETITIVENESS STUDIES

3.16.1 Construction of Tn5 lac Z fusion in the *Azospirillum* sps.

The present investigation was initiated by constructing *Azospirillum* strains ACD-7, ACD-8, ACD-701 and ACD-802 with lac Z fusion and such fusant were used for tracing the inoculated *Azospirillum* strains in the endorhizosphere. *Azospirillum* strains ACD-7, ACD-8, ACD-701 and ACD-802 and *E. coli* strain S-17 mini Tn5 used in the present study were maintained on N free malate medium and Luria agar medium respectively. The antibiotics (Ampicillin) (AMP_{100}) for the 4 strains ACD-7, ACD-8, ACD-701 and ACD-802 and kanamycin (Kan_{50}) for *E. coli* strain S-17 mini Tn5 were determined as the selection pressure. The *E. coli* strain S-17 mini Tn5 has a plasmid which contains promoter less lac Z gene was transferred to *Azospirillum* strains by patch mating (Gerk *et al*., 2000) *Azospirillum* strains and *E. coli* S-17 mini Tn5 were grown overnight in L.B. medium with determined antibiotics. From each of culture 1.5 ml was centrifuged in an Eppendroff tube at 7000 rpm for 3 min and the pellet was washed twice with 0.01 $MgSO_4$, the washed pellets were resuspended in 1 ml L.B. and mixed together (donor recipient 1:1 or 1:2) and centrifuged again. The supernatant was discarded leaving behind 30-40 ml of the medium. The pellet was resuspended in the medium and put as a spot on L.A. plates. After 18 hr, the patch was scarped from the medium and resuspended in L.B. medium Serial dilution of the suspension was placed on L.A. medium containing determined antibiotics. X-gal and IPTG (both 160 µl/100 ml of the L.A. medium fusants which appeared blue on the medium was selected and maintained on the appropriate medium and used in the further work and also its characteristic pellicle growth on the N-free semi solid medium was examined to confirm that it's a derivative of *Azospirillum* strains ACD-7, ACD-8, ACD-701 and ACD-802.

3.16.2 POT CULTURE EXPERIMENT

The pot culture experiment was carried out to study the interaction of wild types and mutants of *Azospirillum* on the endorhizosphere colonization and also competitive ability of the introduced strains in maize plants (DMH-2).

3.16.2.1 Soil characteristics

The physical and chemical properties of the soil are given in the Appendix I. The soil was sterilized by autoclaving at 121℃ @ 15 lbs for 1 hour, surface sterilized plastic pots were filled with 1 kg sterilized soils.

3.16.2.2 Treatments

The experiment was carried out under glasshouse condition and there were totally 8 treatments and each replicated four times.

The observation were taken at 45 DAS, the details of the treatments given below.

T1 - ACD-7
T2 - ACD – 8
T3 - ACD – 701
T4 - ACD – 801
T5 - ACD – 7 + ACD – 8
T6 - ACD – 7 + ACD – 701
T7 - ACD – 8 + ACD – 802
T8 - ACD – 701 + ACD – 802

3.16.2.3 Seed inoculation

Two mutant strains and two wild type strains of *Azospirillum* were grown on Nfb agar with 0.05 g yeast extract per litre for 48 hrs. Growth was scraped and thoroughly mixed with one per cent sterile carboxy methyl cellulose (CMC) suspension. Maize seeds were surface sterilized with sodium hypochlorite (4%) for 25 min and then thoroughly rinsed twice with sterile water. The seeds were then placed in CMC based culture suspension and air-dried overnight by placing in a laminar airflow chamber (LFC). Inoculated seeds were sampled and population of *Azospirillum* per seed was determined on Nfb medium. Seeds coated with only CMC suspension served as control.

3.16.2.4 Sowing and maintenance

The inoculated seeds were sown in pots at the rate of two seeds per pot. Four replications were maintained for each treatment and the pots were kept in the green house. After germination thinning was done to retain one plant per pot and plants were allowed to grow upto 45 DAS with regular watering to maintain optimum moisture throughout.

3.17 ENUMERATION OF *Azospirillum* POPULATION IN RHIOZOSPHERE AND ENDORHIZOSPHERE

Standard plate count technique was used to enumerate the rhizosphere and endorhizosphere *Azospirillum* population, from each treatment 10 g of rhizosphere soil (root bits) collected from each plant of the treatment were serially diluted ten folds ; appropriate dilutions were inoculated (0.1 ml) on LA. + Xgal + IPTG plates and spread uniformly. The plates were incubated at 28°C for 2 days. The development of the colony white and blue indicated the presence of normal and tagged *Azospirillum* respectively. The number of white and blue colony were counted and the per cent competence of the strains was calculated.

3.17.1 Stability of mutants

For checking the stability of the mutants the mutants were streaked on plain L.A. for 10 generations and then from tenth generation it was taken and streaked on L.A. + Azide (40 ppm for ACD-802 and 35 ppm for ACD-701) to check the azide resistance. This was followed for 5 times in fifty generation of mutant *Azospirillum* were checked for Azide resistance stability and finally again checked the nitrogen fixing efficiency in Nfb medium by microkjeldhal method of nitrogen estimation.

3.17.2 Competitiveness of mutants

The competitiveness was found out from the per cent root colonization by the mutants in coinoculation with wild type and mutant in both rhizoplane and endorhizosphere and based on percentage values the competitiveness was interpreted.

3.18 STATISTICAL ANALYSIS OF THE DATA

The data recorded on various characters were subjected to Fisher's method of analysis of variance and interpretation of data as given by Gomez and Gomez (1984). The level of significance used in 'F' test and 't' test was P = 0.01 in laboratory experiments and P = 0.05 in field experiments. Least significance difference (LSD) were calculated whenever the 'F' test was significant.

IV. EXPERIMENTAL RESULTS

Investigation were carried out to study the effect of Azide resistant mutants of *Azospirillum* on nutrient uptake, growth and yield of a major cereal crop *Zea mays*. The results obtained on the investigation are presented in this chapter.

4.1 CHARACTERIZATION AND IDENTIFICATION

4.1.1 Identification

All the five *Azospirillum* strains viz., two mutant ACD-701, ACD-802 and two wild types ACD-7 and ACD-8 along with a reference strain ACD-15 for comparison formed subsurface pellicle in semisolid Nfb malate medium. The pellicle formation was about 1 to 2 mm below the surface of semi solid Nfb medium. All were Gram negative, the isolates formed red and smooth colony on congored medium. The isolates could be easily identified on potato infusion agar a differential medium where the colonies appeared as light pink and dry. Some of them appeared as white and curled and other appeared white and smooth, after seven to nine days of incubation. Colony morphology was also checked on Luria agar and growth was observed on overnight incubation. Some of the isolates appeared as dull pink colonies with lobed edges while others appeared cream with lobed edges.

4.1.2 Microscopic observation

Azospirillum strains were further examined for their Gram-reaction, shape and motility.

Characteristically all the isolates were Gram negative, vibroid and exhibited spiral (Cork-Screw) movement when observed using the hanging drop technique.

4.1.3 Biochemical characteristics

The strains were further examined for physiological properties.

All the strains formed subsurface pellicle in the semi solid medium. All the strains were catalase positive, urease positive and could grow in anaerobic condition in the presence of nitrate. None of the isolate could produce H_2S from cystine or could utilize gelatin.

4.1.3.1 Acid from glucose

None of the 5 strains could not produce acid from glucose.

4.1.3.2 *In vitro* nitrogen fixation

All 5 strains of *Azospirillum* were tested for the total nitrogen fixation by the method given by Humphries (1956). The experiment was conducted in replication of three and the mean values of the replication are presented in Table 4. The total nitrogen fixed was expressed as mg of nitrogen fixed per gram of carbon source utilized.

The mutant strain ACD-802 fixed highest amount of nitrogen followed by ACD-701 in the N free semi solid malate medium i.e., 61.12 and 57.23 mg/g of malate compared to their wild types ACD-8 and ACD-7 that fixed 18.62 and 18.13 mg/g of malate respectively and the reference strain ACD-15 fixed 18.20 mg/g of malate.

Table 4. *In vitro* nitrogen fixation by *Azospirillum* mutants

Strain No.	**N fixed mg/g of malate**
ACD-7	18.13
ACD-8	18.62
ACD-802	61.12
ACD-701	57.23
ACD-15	18.20

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

4.1.4 Production of growth promoting substances

4.1.4.1 IAA

In vitro synthesis of IAA by the two azide resistant mutants their wild types and one standard check of *Azospirillum* was examined on a medium with tryptophan as precursor. All isolate could produce IAA, ranging from 7.36 mg/100 ml to 42.34 mg/100 ml. Among all the isolates examined for IAA production the mutant strain ACD-802 produced maximum amount of IAA and was superior over all other isolates followed by ACD-701 that produced 39.53 mg/100 ml compared to standard ACD-15 the two wild types also showed higher production of IAA. The detailed results are presented in Table 5.

4.1.4.2 Gibberellic acid (GA) production

Mutant ACD-802 produced highest amount of GA (4.16 mg/25 ml) followed by mutant ACD-701 (3.92 mg/2 ml), much variation was not seen in the standard ACD-15 and the two wild types ACD-8 and ACD-7.

4.2 CHARACTERIZATION OF MUTANTS

4.2.1 Sodium azide resistance

ACD-802 the mutant strain recorded the highest resistance to sodium azide upto 40 ppm followed by mutant ACD-701 that showed a resistance upto 35 ppm. Both the wild types ACD-8 and ACD-7 were resistant upto 25 ppm and reference strain ACD-15 was resistant upto only 15 ppm concentration of sodium azide.

4.3 FIELD STUDIES

Seed treatment of *Azospirillum brasilense* strains ACD-8 and ACD-7 along with their mutants ACD-802 and ACD-701 with two different fertilizer N-levels i.e., 75 per cent RDN and 100 per cent RDN showed a significant influence on different plant growth parameters, nutrient uptake and yield component at different stages of maize crop growth.

4.3.1 Plant height

Data on plant height are presented in Table 6. Results indicated that application of *Azospirillum* with two levels of nitrogen significantly increased the plant height of maize plant at 60, 90 DAS and at harvest of crop growth.

Significantly higher plant height of 177.35, 180.84 and 183.40 were recorded in T4 (ACD-804 + 100% RDN) followed by T3 (ACD-802 + 75% RDN), 176.21, 178.49 and 182.04 cm, T8 (ACD-701 + 100% RDN), 173.89, 178.47 and 183.02 and T7 (ACD 701 + 75%RDN) which were statistically on par with each other. And lower plant height was recorded in the treatment T9 (ACD-7 + 75% RDN) 155.85, 162.65 and 164.80 and the least plant height of 145.69, 152.69 and 153.99 were recorded in T11 (uninoculated control + 100% RDN), at 30, 60, 90 DAS and at harvest respectively.

4.3.2 Total dry matter

Significant difference in total dry matter production were observed due to seed treatment of mutant and wild type strains of *Azospirillum* with two levels of nitrogen. The data on the total dry matter are presented in the Table 7.

Significantly higher total dry weight of 121.48, 250.20 and 286.45 g per plant in T4 (ACD-802 + 100% RDN) and T3 (ACD-802 + 75% RDN) 120.29, 248.44 and 283.55 g per plant which was on par with each other compared to both the wild types the mutant showed significantly higher dry matter accumulation. The treatment T7 (ACD-701 + 75 RDN) and T8

Table 5. Production of growth promoting substance

Strain No.	IAA (µg/100 ml)	GA (µg/25 ml)
ACD-7	29.80	2.84
ACD-8	28.70	3.02
ACD-701	39.53	3.92
ACD-802	42.34	4.16
ACD-15	7.36	2.92

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 6. Plant height of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Plant height (cm/plant)			
	30	**60**	**90**	**Harvest**
T1 – ACD-15 + 75% RDN	34.61	162.71	160.88	164.60
T2 - ACD-15 + 100% RDN	35.97	161.93	163.88	166.56
T3 – ACD-802 + 75% RDN	38.12	176.21	178.49	182.04
T4 – ACD-802 + 100% RDN	38.71	177.35	180.84	183.40
T5 – ACD-8 + 75% RDN	35.74	152.03	160.50	164.43
T6 - ACD-8 + 100% RDN	36.60	154.42	167.31	167.85
T7 – ACD-701 + 75% RDN	37.14	172.62	177.46	182.08
T8 – ACD-701 + 100% RDN	37.23	173.89	176.47	183.02
T9 – ACD-7 + 75% RDN	35.63	155.85	162.65	164.80
T10 - ACD-7 + 100% RDN	35.83	153.93	164.27	168.15
T11 – UIC + 100% RDN	31.94	145.69	152.69	153.99
S.Em±	4.814	6.831	6.488	6.653
CD at 5%	NS	20.150	19.12	19.623

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 7. Total dry matter of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Total dry matter (g/plant)			
	30	60	90	Harvest
T1 – ACD-15 + 75% RDN	14.47	110.26	225.23	253.23
T2 - ACD-15 + 100% RDN	14.37	109.58	223.23	250.19
T3 – ACD-802 + 75% RDN	15.23	120.29	248.44	283.55
T4 – ACD-802 + 100% RDN	16.30	121.48	250.20	286.45
T5 – ACD-8 + 75% RDN	14.31	103.83	229.34	261.44
T6 - ACD-8 + 100% RDN	14.11	97.61	225.27	259.31
T7 – ACD-701 + 75% RDN	15.53	112.92	236.27	249.24
T8 – ACD-701 + 100% RDN	15.29	114.00	238.47	250.32
T9 – ACD-7 + 75% RDN	13.96	97.31	198.36	230.08
T10 - ACD-7 + 100% RDN	14.67	108.25	193.58	230.92
T11 – UIC + 100% RDN	12.11	81.24	175.21	200.39
S.Em±	0.781	6.104	7.016	6.805
CD at 5%	NS	18.003	20.693	20.071

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Plate1: General view of maize crop

(ACD-701 + 100% RDN) were on par with the treatment T4 at 60 and 90 DAS and at harvest respectively. The least dry weight of 12.11, 81.24, 175.21 and 200.39 g per plant were observed in T11 (Uninoculated control + 100% RDN) at 60, 90 DAS and at harvest, respectively.

4.3.3 Number of leaves

There was no significant difference recorded in number of leaves per plant due to inoculation of *Azospirillum* strains and two levels of nitrogen through out the crop growth (Table 8).

4.3.4 Chlorophyll content

The chlorophyll content in maize crop at different stage of crop growth due to inoculation of *Azospirillum* wild types and their sodium azide resistant mutants with different dosage of N-fertilizer is presented in Table 9.

Except 30 DAS, the chlorophyll content in maize differ significantly at all other stages of crop growth.

Chlorophyll content increased gradually with crop growth and decline at 90 DAS and at harvest. At 60 DAS, the highest chlorophyll content was recorded in treatment T4 i.e., ACD-802 + 100% RDN followed by T8 (3.753), T3 (3.700) and T7 (3.689) which were on par with each other and lowest value was registered in T11 control (2.700) which was on par with T2 (2.873) and then followed by T1 (3.202). Similar trend was observed at 90 DAS and also at harvest and compared to wild types the treatments containing mutant strains were significantly superior.

4.3.5 Shoot N and root N uptake (mg/plant)

Application of Azide resistant mutants of *Azospirillum* as seed inoculant along with two different dose of nitrogen fertilizer showed significant increase in shoot nitrogen uptake and root nitrogen uptake content in all the treatment over inoculated and uninoculated control. Data on uptake of nitrogen in shoot and root are presented in Table 10 and 11. Results indicated the application of mutant *Azospirillum* with N fertilizer significantly increased the shoot and root N uptake by maize plant at all stages of crop growth (30, 60, 90 DAS and at harvest).

The maximum shoot N uptake was recorded in treatment T4 (344, 1386, 2228, 1743 mg/plant), T3 (322, 1312, 2167, 1655 mg/plant) and T8 (307, 1301, 2145, 1639 mg/plant) which were on par with each other. And a minimum shoot N uptake (200, 1019, 1469, 1402 mg/plant) was recorded in T11 uninoculated control at 30, 60, 90 DAS and at harvest.

Similarly, the maximum N uptake by root was recorded in treatment T4 (86.99, 181.66, 202.62, 196.60 mg/root) and T3 (78.36, 150.20, 180.72, 165.00 mg/root) and minimum uptake was recorded in T11 (59.70, 66.74, 100.62, 79.20 mg/root) and T9 (60.00, 87.00, 116.01, 93.00 mg/root) followed by T10 (65.00, 113.90, 137.3, 119.40 mg/root) at 30, 60, 90 DAS and at harvest respectively.

4.3.6 Stem girth

The data on stem girth of maize plant differed significantly at all the stage of plant growth (Table 12).

Significantly highest stem girth was recorded in treatment T4 (ACD 802 + 100%) 2.27, 2.73, 2.79 and 2.84 cm/plant followed by T3 (ACD 802 + 75%N) 2.22, 2.65, 2.71 and 2.77 cm plant, T8 (ACD 701 + 100%N) 2.22, 2.62, 2.68 and 2.70 and T7 (ACD 701 + 75%N)

Table 8. Number of leaves of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Number of leaves		
	30	**60**	**90**
T1 – ACD-15 + 75% RDN	6.83	13.86	13.10
T2 - ACD-15 + 100% RDN	6.73	13.94	12.93
T3 – ACD-802 + 75% RDN	7.52	14.23	12.74
T4 – ACD-802 + 100% RDN	7.50	14.35	13.53
T5 – ACD-8 + 75% RDN	6.80	13.50	13.02
T6 - ACD-8 + 100% RDN	6.80	13.82	13.11
T7 – ACD-701 + 75% RDN	7.46	14.28	13.72
T8 – ACD-701 + 100% RDN	7.23	14.35	12.01
T9 – ACD-7 + 75% RDN	6.90	13.75	12.22
T10 - ACD-7 + 100% RDN	6.86	13.71	12.19
T11 – UIC + 100% RDN	6.27	13.11	12.32
S.Em±	0.397	0.518	0.615
CD at 5%	NS	NS	NS

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 9. Total chlorophyll content of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Total chlorophyll content (mg/g)			
	30	60	90	Harvest
T1 – ACD-15 + 75% RDN	2.272	3.202	1.376	0.844
T2 - ACD-15 + 100% RDN	2.171	2.873	1.210	0.711
T3 – ACD-802 + 75% RDN	2.340	3.700	1.717	1.198
T4 – ACD-802 + 100% RDN	2.402	4.060	1.842	1.247
T5 – ACD-8 + 75% RDN	2.270	3.217	1.387	0.861
T6 - ACD-8 + 100% RDN	2.298	3.340	1.597	0.999
T7 – ACD-701 + 75% RDN	2.333	3.689	1.697	1.116
T8 – ACD-701 + 100% RDN	2.372	3.753	1.730	1.210
T9 – ACD-7 + 75% RDN	2.286	3.314	1.498	0.901
T10 - ACD-7 + 100% RDN	2.305	3.631	1.521	0.976
T11 – UIC + 100% RDN	2.005	2.700	1.004	0.623
S.Em±	0.096	0.131	0.068	0.046
CD at 5%	NS	0.388	0.202	0.138

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 10. Shoot N uptake of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Shoot N uptake (mg/plant)			
	30	**60**	**90**	**Harvest**
T1 – ACD-15 + 75% RDN	250	1156	1867	1511
T2 - ACD-15 + 100% RDN	267	1246	2051	1561
T3 – ACD-802 + 75% RDN	322	1312	2167	1655
T4 – ACD-802 + 100% RDN	344	1386	2228	1743
T5 – ACD-8 + 75% RDN	270	1254	2047	1566
T6 - ACD-8 + 100% RDN	274	1269	2054	1573
T7 – ACD-701 + 75% RDN	298	1262	2046	1589
T8 – ACD-701 + 100% RDN	307	1301	2145	1639
T9 – ACD-7 + 75% RDN	227	1036	1637	1454
T10 - ACD-7 + 100% RDN	248	1184	1912	1499
T11 – UIC + 100% RDN	200	1019	1469	1402
S.Em±	14.72	36.46	57.73	56.81
CD at 5%	42.51	108.27	171.46	168.74

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 11. Root N uptake of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Root N uptake (mg/plant)			
	30	**60**	**90**	**Harvest**
T1 – ACD-15 + 75% RDN	69.70	119.27	139.42	121.00
T2 - ACD-15 + 100% RDN	74.10	140.59	153.13	147.60
T3 – ACD-802 + 75% RDN	80.26	167.00	182.00	178.00
T4 – ACD-802 + 100% RDN	86.99	181.66	202.62	196.60
T5 – ACD-8 + 75% RDN	74.42	142.32	159.43	151.20
T6 - ACD-8 + 100% RDN	74.92	146.00	161.32	159.00
T7 – ACD-701 + 75% RDN	76.32	147.98	170.41	162.36
T8 – ACD-701 + 100% RDN	78.36	150.20	180.72	165.00
T9 – ACD-7 + 75% RDN	60.00	87.00	116.01	93.00
T10 - ACD-7 + 100% RDN	68.00	113.90	137.33	119.40
T11 – UIC + 100% RDN	59.70	66.74	100.82	79.20
S.Em±	2.69	6.92	7.07	5.97
CD at 5%	7.98	20.76	21.21	17.96

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

2.20, 2.53, 2.57 and 2.60 cm/plant which were on par to each other and least stem girth was recorded in T11 (uninoculated control + 100% N) 1.67, 2.07, 2.10 and 2.40 cm/plant at 30, 60, 90 DAS and at harvest respectively.

Compared to wild types and the standard control superior stem girth was observed in the treatments containing the mutant strains.

4.4.7 Grain N, cob and grain parameters of maize

Grain N (mg/cob), cob length (cm), cob weight (g) and 100 grain weight (g) of maize at harvest were significantly influenced by inoculation of *Azospirillum* with two levels of nitrogen, it is presented in the Table 13 and 14.

The maximum Grain N was recorded in treatment T4 (ACD-802 + 100% RDN) 643 mg per cob, T3 (ACD-802 + 75% RDN) 612 mg per cob, T8 (ACD-701 + 100% RDN) 609 mg per cob and T7 (ACD-701 + 75% RDN) 599 mg per cob which were on par with each other and the minimum Grain N of 501 mg per cob in T9 (ACD-701 + 75% RDN) and followed by T11 (uninoculated control + 100% RDN) 473 mg per cob.

Cob length ranged from 15.68 cm to 17.59 but, no significant treatmental differences were observed in cob length.

Highest cob weight was recorded in the treatment T4 (ACD-802 + 100% RDN) 189.49 g followed by T3 (ACD-802 + 75% RDN) 186.43 g, T8 (ACD-701 + 100% RDN) 183.27 g and T7 (ACD-701 + 75% RDN) 181.23 g which were on par with each other.

Similar trend was recorded in 100 grain weight highest weight was recorded in T4 (ACD-802 + 100% RDN), 24.29 g and lowest was found in uninoculated control + 100% RDN (17.41 g).

4.4.8 Grain yield and stover yield (q/ha)

The data on grain yield and stover yield as influenced by seed inoculation of azide resistant mutants of *Azospirillum* with two levels of nitrogen is given in the Table 15.

Upon inoculation significant variation of grain yield and stover yield was noticed. Compared to the wild type, reference and uninoculated control the results due to mutants were superior.

The maximum grain yield was recorded in T4, T3 T8 and T7 (45.11, 44.37, 43.75 and 43.52 q/ha) which were on par with each other and similar trend was observed in stover yield where the maximum tover yield was recorded in treatments T4, T3, T8 and T7 (64.74, 63.55, 61.99 and 61.63 q/ha) which were on par with each other.

4.5 POPULATION DYNAMICS OF AZOSPIRILLUM

4.5.1 Population dynamics of endorhizosphere *Azospirillum* sp.

Endophytic colonization of *Azospirillum* strain as enumerated by most probable number method, due to inoculation of *Azospirillum* strains in combination of two levels of nitrogen is presented in Table 16. Mean *Azospirillum* colonization in the maize root system increased gradually with the crop growth and reached its peak by 60 DAS. But there after the population of the endophytic declines.

At 30 DAS, treatment T4 (ACD-802 + 100% RDN) recorded highest root colonization 2.89 x 10^4 MPN/g dry root followed by treatment T9 (ACD-701 + 100% RDN), 2.49 X 10^4

Table 12. Stem girth of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Stem girth (cm)			
	30	60	90	Harvest
T1 – ACD-15 + 75% RDN	1.99	2.22	2.29	2.32
T2 - ACD-15 + 100% RDN	2.04	2.17	2.27	2.30
T3 – ACD-802 + 75% RDN	2.22	2.65	2.71	2.77
T4 – ACD-802 + 100% RDN	2.27	2.73	2.79	2.84
T5 – ACD-8 + 75% RDN	1.96	2.19	2.27	2.28
T6 - ACD-8 + 100% RDN	2.00	2.20	2.27	2.27
T7 – ACD-701 + 75% RDN	2.20	2.53	2.57	2.60
T8 – ACD-701 + 100% RDN	2.22	2.62	2.68	2.70
T9 – ACD-7 + 75% RDN	1.99	2.21	2.26	2.27
T10 - ACD-7 + 100% RDN	1.98	2.21	2.27	2.28
T11 – UIC + 100% RDN	1.67	2.07	2.10	2.40
S.Em±	0.087	0.080	0.072	0.078
CD at 5%	0.258	0.236	0.213	0.227

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 13. Test weight (100-seed) and grain N of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Test weight (g)	Grain N (mg/plant)
T1 – ACD-15 + 75% RDN	22.43	566
T2 - ACD-15 + 100% RDN	22.42	578
T3 – ACD-802 + 75% RDN	23.70	612
T4 – ACD-802 + 100% RDN	24.29	643
T5 – ACD-8 + 75% RDN	22.25	576
T6 - ACD-8 + 100% RDN	21.50	583
T7 – ACD-701 + 75% RDN	23.10	599
T8 – ACD-701 + 100% RDN	24.18	609
T9 – ACD-7 + 75% RDN	19.40	501
T10 - ACD-7 + 100% RDN	19.80	574
T11 – UIC + 100% RDN	17.41	473
S.Em±	1.174	16.715
CD at 5%	3.463	49.301

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 14. Cob length and cob weight of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Cob length (cm)	Cob weight (g)
T1 – ACD-15 + 75% RDN	17.27	171.36
T2 - ACD-15 + 100% RDN	17.15	172.40
T3 – ACD-802 + 75% RDN	17.58	186.43
T4 – ACD-802 + 100% RDN	17.59	189.49
T5 – ACD-8 + 75% RDN	17.09	167.29
T6 - ACD-8 + 100% RDN	17.29	172.25
T7 – ACD-701 + 75% RDN	17.41	181.23
T8 – ACD-701 + 100% RDN	17.47	183.27
T9 – ACD-7 + 75% RDN	16.32	170.02
T10 - ACD-7 + 100% RDN	16.61	171.27
T11 – UIC + 100% RDN	15.68	151.75
S.Em±	0.735	5.627
CD at 5%	NS	16.596

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 15. Grain yield and stalk yield of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Grain yield (q/ha)	Stalk (q/ha)
T1 – ACD-15 + 75% RDN	40.45	56.25
T2 - ACD-15 + 100% RDN	40.35	57.90
T3 – ACD-802 + 75% RDN	44.37	63.55
T4 – ACD-802 + 100% RDN	45.11	64.74
T5 – ACD-8 + 75% RDN	39.39	56.04
T6 - ACD-8 + 100% RDN	40.15	56.91
T7 – ACD-701 + 75% RDN	43.52	61.63
T8 – ACD-701 + 100% RDN	43.75	61.99
T9 – ACD-7 + 75% RDN	39.32	57.77
T10 - ACD-7 + 100% RDN	39.25	58.37
T11 – UIC + 100% RDN	31.80	48.70
S.Em±	1.225	1.691
CD at 5%	3.612	4.987

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 16. Population dynamics of endorhizosphere 10^4 MPN g dry $root^{-1}$ of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Endorhizosphere 10^4 MPN g dry $root^{-1}$			
	30	60	90	Harvest
T1 – ACD-15 + 75% RDN	1.20	20.20	14.24	11.82
T2 - ACD-15 + 100% RDN	1.70	22.15	15.00	12.10
T3 – ACD-802 + 75% RDN	2.01	25.68	17.50	14.10
T4 – ACD-802 + 100% RDN	2.89	26.20	19.00	15.00
T5 – ACD-8 + 75% RDN	1.30	23.05	16.70	12.74
T6 - ACD-8 + 100% RDN	1.86	21.11	14.89	11.88
T7 – ACD-701 + 75% RDN	2.21	23.01	15.30	12.70
T8 – ACD-701 + 100% RDN	2.49	25.03	17.71	14.00
T9 – ACD-7 + 75% RDN	1.42	19.99	12.89	10.55
T10 - ACD-7 + 100% RDN	1.61	20.01	13.10	10.36
T11 – UIC + 100% RDN	0.60	4.59	9.90	6.72
S.Em±	0.091	0.912	0.620	0.761
CD at 5%	0.269	2.689	1.830	2.243

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

MPN/g dry root and T8 (ACD-701 + 75% RDN) 2.21 x 10^4 MPN) g dry root. The treatment T4 was significantly superior over all the other treatments and the lowest root colonization was observed in treatment T11 (0.60 x 10^4 MPN/g dry root).

Similar results were observed at 60 DAS the highest root colonization was in treatment T4 (26.20 x 10^4 MPN/g dry root) followed by T3 (25.68 x 10^4 MPN/g dry root) and treatment T8 (25.03 x 10^4 MPN/g dry root) all these three treatments were on par with each other, but significantly superior over the all other treatments and lowest root colonization was observed in treatment T11 (14.59 x 10^4 MPN/g dry root).

Similarly at 90 DAS and at harvest the highest root colonization was seen in treatment T4 (19.00 and 15.00 x 10^4 MPN/g dry root) and the lowest number was recorded in uninoculated control i.e., T11 (9.90 and 6.72 10^4 MPN/g dry root).

4.5.2 Population dynamics of *Azospirillum* in Rhizosphere

In general, the treatments differed significantly in the *Azospirillum* population at all the growth stages (Table 17).

Among the two mutants highest rhizosphere population was found in the treatment inoculated with ACD-802 + 100 per cent N of RDN (19.56 x 10^4 MPN/g dry soil) and least in case of ACD-701 + 75 per cent N of RD (15.95 x 10^4 MPN/g dry soil).

Similarly at 60, 90 DAS and at harvest the highest rhizosphere population was found in treatment T4 (45.37, 60.47 and 23.14 x 10^4 MPN/g dry soil) and the least population was found in treatment T11 is uninoculated control + 100% RDN (28.15, 42.28 and 11.72 x 10^4 MPN/g dry soil).

4.6 STABILITY AND COMPETITIVENESS

4.6.1 Lac Z gene tagging to *Azospirillum*

All the 4 strains of *Azospirillum* viz., two wild types ACD-7 and ACD-8 and the two mutants ACD-701 and ACD-802 were tagged with Lac Z gene by inserting Lac Z using the *E. coli* strain S-17 mini Tn5. All four strains were successfully fused with the Lac Z gene and the transformed *Azospirillum* strains formed blue colour colonies on the LA + Xgal (160 µl/100 ml) + IPTG (160 µl/100 ml) plates. The blue colour indicated the presence of gene in the strains and was sued as an indicator marker to trace back the strains.

4.7 POT CULTURE EXPERIMENTS

The results of pot culture experiment conducted during August 2005, to study competitiveness of azide resistant mutants of *Azospirillum* on maize plants are presented here.

4.7.1 *Azospirillum* population on rhizoplane

In the co inoculation of the wild type and mutant strains the rhizoplane population was significantly superior over the single inoculation treatments and the highest rhizoplane population was seen in the treatment T8 (ACD-701 + ACD-802) 31.75 x 10^4 cfu per 10 g of dry roots followed by T6 (ACD-7 + ACD-701) 30.57 x 10^4 cfu/10 g roots and T7 (ACD-8 + ACD-802) 30.00 x 10^4 cfu/10 g of roots where all the treatments were on par with each other (Table 18).

Table 17. Population dynamics of rhizosphere 10^4 MPN g dry $soil^{-1}$ of maize at different growth stages as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Rhizosphere 10^4 MPN g dry $root^{-1}$			
	30	60	90	Harvest
T1 – ACD-15 + 75% RDN	14.46	40.66	54.78	18.15
T2 - ACD-15 + 100% RDN	14.91	40.29	54.90	17.95
T3 – ACD-802 + 75% RDN	18.20	42.96	58.70	21.63
T4 – ACD-802 + 100% RDN	19.56	45.37	60.47	23.14
T5 – ACD-8 + 75% RDN	10.83	36.62	52.86	14.59
T6 - ACD-8 + 100% RDN	13.46	38.43	52.63	16.59
T7 – ACD-701 + 75% RDN	15.95	41.80	56.82	19.72
T8 – ACD-701 + 100% RDN	16.32	41.89	56.98	19.83
T9 – ACD-7 + 75% RDN	11.52	36.65	51.15	13.56
T10 - ACD-7 + 100% RDN	11.67	37.11	51.35	13.89
T11 – UIC + 100% RDN	10.39	28.15	42.28	11.72
S.Em±	0.552	1.259	1.611	1.319
CD at 5%	1.629	3.714	4.753	3.890

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

4.7.2 *Azospirillum* population in endorhizosphere

In Endorhizosphere the coinoculation of the mutant and wild type and the mutant and mutant was significantly superior over the other single inoculation treatment among the treatments the maximum population was recorded in the treatment T8 (ACD-701 + ACD-802) 19.25 x 10^3 cfu/10 g dry root followed by T7 (ACD-8 + ACD-701) 18.25 x 10^3 cfu/10 g dry root which were on par with each other and the least population was seen in T1 (ACD-7) and T2 (ACD-8) which were having the same population of 11.5 x 10^3 cfu/10 g dry root.

Table 18. Population on Rhizoplane and in Endorhizosphere of maize at 45 DAS as influenced by seed treatment of azide resistant mutants of *Azospirillum*

Treatments	Rhizoplane $X10^{-4}$ CFU 10 g dry $root^{-1}$	Endorhizosphere $X10^{-4}$ CFU 10 g dry $root^{-1}$
T1 – ACD-7	20.00	11.50
T2 - ACD-8	21.75	11.50
T3 – ASD-701	22.50	12.50
T4 – ASD-802	22.75	12.75
T5 – ASD-7 + ACD-8	28.00	17.75
T6 - ASD-7 + ACD-701	30.75	18.25
T7 – ASD-802 + ACD-8	30.00	18.75
T8 – ASD-701 + ACD-802	31.75	19.25
S.Em±	0.550	0.348
CD at 5%	2.203	1.393

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

Table 19. Intrinsic antibiotic resistance of azide resistance mutants of *Azospirillum*

Strains	Nalidixic acid (ppm)			Kanamycin (ppm)			Chloromphenicol (ppm)			Ampicilin (ppm)			Gentamycin (ppm)			Spectinomycin (ppm)			Hygromycni (ppm)			Tetracycline (ppm)			Streptomycin (ppm)			Control
	10	20	30	25	50	100	10	20	25	50	100	150	50	100	150	10	20	30	50	100	150	10	20	25	50	75	100	
ACD-7	++	++	+	++	+	+/-	+/-	-	-	++	-	+/-	-	-	-	++	+	-	++	++	+	++	+	-	+	-	-	++
ACD-701	++	+	-	++	-	+/-	+	-	-	++	-	+/-	-	-	-	++	+	-	+	+/-	-	++	+	-	+	-	-	++
ACD-8	++	++	-	++	+	+/-	+	-	-	++	-	+/-	-	-	-	++	+	-	++	+	-	++	+/-	-	+	-	-	++
ACD-802	++	+	-	++	+	+/-	+	-	-	++	-	+/-	-	-	-	++	+	-	+	+/-	-	++	+	-	+	-	-	++
ACD-15	++	-	-	++	+	-	+	-	-	++	-	-	-	-	-	++	+/-	-	+	+	-	++	+	-	+	-	-	++

Note :
ACD – 802 and ACD- 701 mutants
ACD-8 and ACD- 7 wild types.
ACD – 15 Reference strain.

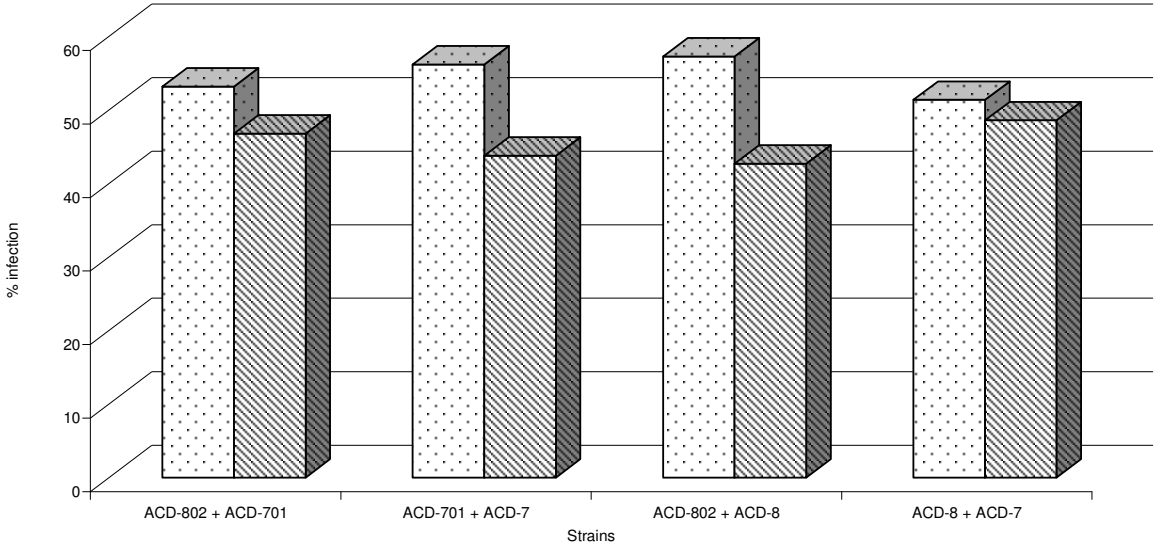

Fig. 2: Competitive colonization rhizoplane

Fig.2. Competitive colonization rhizoplane

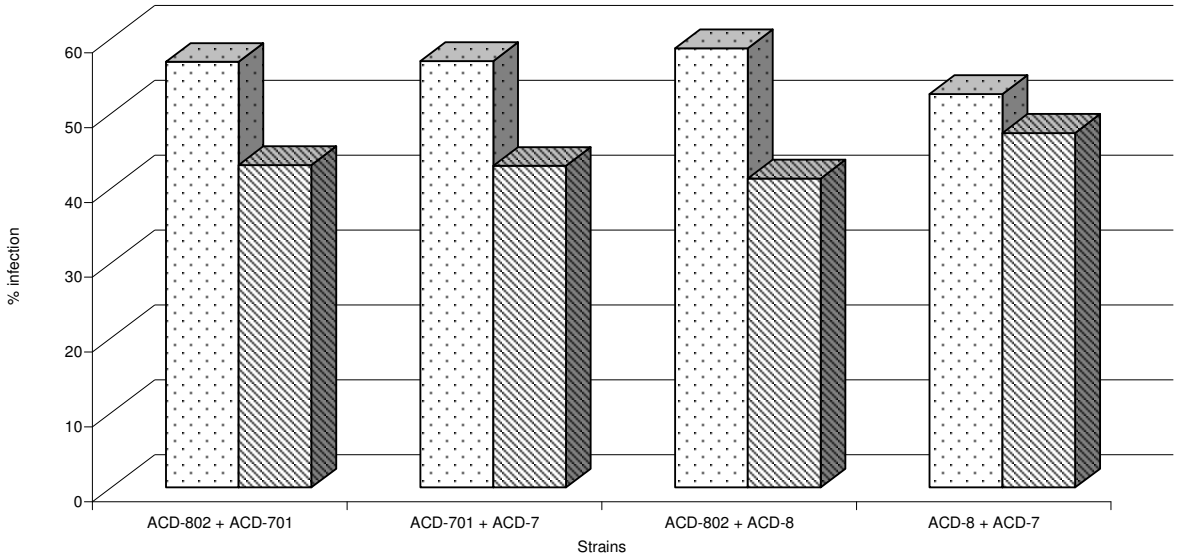

Fig. 3: Competitive colonization endorhizosphere

Fig.3. Competitive colonization endorhizosphere

4.8 COMPETITIVE COLONIZATION ENDORHIZOSPHERE

The dual inoculation of the Lac Z gene tagged mutant strain with the wild type and the mutant indicated that among the two mutants ACD-802 was found to be more competitive than the both the wild types and mutant ACD-701. Against the mutant ACD-701, the ACD-802 showed 53.2 per cent endorhizosphere colonization whereas ACD-701 showed only 46.8 per cent root colonization among the wild types ACD-8 was found more competitive than the ACD-7 it showed 51.4 per cent root colonization and 48.6 per cent respectively, but in the single inoculation the results were on par to the wild types and the least population was seen in the two wild types (Fig. 2).

4.8.1 Competitive colonization assay (Rhizoplane)

The pair wise inoculation of the Lac Z tagged mutant strain with the wild type and the mutant showed that between the two mutants. ACD-802 was more competitive than the both the wild types ACD-7 and ACD-8.

It was also found that it was more competitive than the mutant strain ACD-701 against which it showed 56.9 per cent root colonization and against the wild type ACD-8 it showed 58.7 per cent colonization followed by the mutant strain ACD-701 that showed 57 per cent root colonization against the wild type ACD-7.

Between the two wild types the strain ACD-8 was more competitive than the second wild type that is ACD-7 and showed 52.6 per cent root colonization against ACD-7 (Fig.3).

V. DISCUSSION

The production of chemical nitrogenous fertilizer is mainly based on the non-renewable energy sources. The utilization of fossil energy based on fertilizers also triggers pollution. The above mentioned factor coupled with a requirement for high protein plant food have led to renewed efforts in the field of nitrogen fixation research. The ability to fix dinitrogen is limited to the prokaryotic microorganisms and these diazotrophs can be classified as symbiotic, free living and associative nitrogen fixers.

Azospirillum besides nitrogen fixation, is also known to synthesize growth promoting substances, *Azospirillum* has been closely associated with the roots of cereals and help in the fixation of nitrogen.

Many workers have reported that the increase in dry matter content, growth and grain yield of maize were obtained after inoculation with *Azospirillum* (Casanovas *et al*., 2000; Woodard and Bly, 2000). For harvesting the potential of the biologically fixed nitrogen, an effective inoculant of *Azospirillum* is required. Natural selection and mutagenesis provide efficient tool to develop an effective inoculant. Hence, the present study attempted to use these tools to analyse the possibility to arrive at better strains both through selection and mutagenesis. The results obtained are discussed, hereunder.

5.1 CHARACTERIZATION OF AZOSPIRILLUM STRIANS

The isolates made from roots of maize forming the subsurface pellicle in N-free semisolid malate medium is often taken to be an absolute proof of the presence of *Azospirillum* spp. (Okon *et al*., 1977). However, the species of *Bacillus* and *Herbaspirillum* are also known to form pellicle (Kreig and Dobereinier, 1984). Therefore, the pellicle formation, only may not be considered as the only criteria for its identification.

The mutants and wild type strains of *Azospirillum* appeared as light pink and dry, some as white and curled and also are appeared as white and smooth on BMS agar after seven days of incubation. The strains on NFb medium were small, pale green and dense. The morphological characteristics of the strains in comparison with the standard culture of *Azospirillum* ACD-15 showed similarity and were in accordance with the description of *Azospirillum* spp. given by Kreig and Dobereiner (1984) and Tarrand *et al.* (1978). The isolates were further differentiated from *Herbaspirillum* and *Bacillus* species by plating them on congo red medium. The *Azospirillum* mutant and wild type strains appeared as small, smooth and red colour after five to six days of incubation later turning to scarlet. The colony morphology of the strains were in accordance with the description of *Azospirillum* given by Rodriguez-Caceres (1982).

Microscopic examination of these isolates revealed that they were Gram negative, vibroid in shape and showed spirilar movement. These characters were similar to the reference culture and to the genus *Azospirillum* as described by Tarrand *et al.* (1978) and Kreig and Dobereiner (1984). Further, the presence of PHB granules let accordance to the identity of the strains as *Azospirillum*.

5.2 SCREENING OF AZOSPIRILLUM ISOLATES FOR N2-FIXING EFFICIENCY

Total nitrogen fixation by different strains was estimated *in vitro* by Microkjeldhal method (Jackson, 1973) in N-free semi solid malate medium. In the present investigation the total nitrogen fixation ranged from 18.13 mg of N/g to 61.12 mg of N/g of carbon source utilized.

The highest amount of nitrogen was fixed by the mutant strain ACD-802 followed by ACD-701 i.e., 61.12 and 57.23 mg/g of malate respectively and in wild types, ACD-8 fixed

more N than ACD-7 (18.62 and 18.13 mg/g of malate respectively). Reports of nitrogen fixing efficiency of *Azospirillum* strains isolated from grasses ranged from as low as 3.4 mg (Quintero and Garza, 1978) to as high as 83.3 mg of nitrogen fixed per gram of carbon source consumed (Pedrosa *et al*., 1980). Maheshkumar (1997) reported that 15.68 to 22.40 mg nitrogen per gram of carbon source was fixed by *Azospirillum* spp. Shubha (1999) found that 2.76 to 24.80 mg nitrogen was fixed per gram of carbon by *Azospirillum* spp. Isolated from wheat roots. The two mutants ACD-802 and ACD-701 showed higher nitrogenase activity and N fixed g^{-1} of malate *in vitro* against their wild types. These results are in accordance with the reports of Ram *et al.* (1978), Bala and Gaur (1997) and Dayamani (2003).

5.2.1 Production of growth promoting substances

Besides nitrogen fixation, *Azospirillum* also known to produce plant growth promoting substances which in turn affect the root growth, increased N uptake and yield (Tien *et al*., 1979). In the present investigation the isolates were screened for IAA and GA production.

IAA produced by *Azospirillum* isolates ranged from 7.36-µg/100 ml in ACD-15 to 42.34-µg/100 ml in ACD-802. The GA production ranged from 2.92-to 4.16-µg/25 ml. Among all the 5 strains maximum IAA and GA production was seen in ACD-802. Fallick *et al.* (1989) observed 32 to 40 µg/ml IAA production by 15 *Azospirillum* strains. Whereas, Veena (1999) reported that *Azospirillum* strains produced 16.20 to 40.21 µg IAA and 0.56 to 1.84 µg GA 25 ml^{-1}. Gadagi (1999) observed 1.12 to 38.12 µg/100 ml IAA production and 0.12 to 4.32 µg/25 ml GA production by *Azospirillum* strains. Production of IAA and GA by *Azospirillum* strains are also reported by other workers (Tien *et al*., 1979; Hartmann *et al*., 1983; Barbieri *et al*. , 1986; Fallick *et al*., 1989 and Botini *et al*., 1989).

5.3 MICROBIOLOGICAL ASSAY

5.3.1 Rhizosphere *Azospirillum* sp.

Azospirillum sp. can colonize roots externally and internally. In observed colonization, the bacteria form mainly small aggregates, although many single cells may also be scattered on the root surface. These externally colonizing bacteria are embedded on the mucigel layers of the root surface (Schank *et al*., 1979 and Basham *et al*., 1986).

In the present investigation, highest population of *Azospirillum* was found in the treatment inoculated with ACD-802 + 100 per cent N RD at 60 DAS (45.37 x 10^4 g-1 dry soil). This may be due to increased supply of root exudates at flowering stage (60 DAS). *Azospirillum* sp. being nutritionally versatile, can have many alternative metabolic pathways which allow it to consume a wide variety of organic acids, sugars and amino acids available in the rhizosphere from plant and microbial biomass (Okon, 1983).

5.3.2 Endorhizosphere *Azospirillum* sp.

Azospirillum is capable of internal colonization of the intercellular spaces of cortex (Patriquin and Dobereiner, 1978).

In the present investigation, maximum population of endorhizosphere. *Azospirillum* sp. was recorded in the treatment inoculated with ACD-802 + 100 per cent N of RD (26.20 x 10^4 g^{-1} of dry root) at 60 DAS. This may be due to the production of growth promoting substances. This indicates that the recommended dose of nitrogenous fertilizers play an important role in colonization of *Azospirillum* sp. in the rhiozosphere and internal colonization of the intercellular spaces of the cortex (Patriquin and Dobereiner, 1978).

The results obtained in the present investigation also indicated a significant increase in *Azospirillum* population in rhizosphere and endorhizosphere of maize due to inoculation of N fertilizer application. Among the various stages of plant growth examined, the population of N, fixes was maximum at 60 DAS and declined at 90 DAS and at harvest. It is likely that the root biomass were higher at 60 DAS and decomposed thereafter causing a decline in

population at harvest. The observed variation in the rhizosphere population of *Azospirillum* due to plant age agreed with the earlier findings of Roviva and Doney (1974) and Neelakanthan and Rangaswami (1965).

5.4 PERFORMANCE OF AZIDE RESISTANT MUTANTS ON NUTRIENT UPTAKE, GROWTH AND YIELD OF MAIZE

The parameter viz., plant height, root length, dry matter yield, nutrient uptake and yield component of maize crop were significantly increased due to application of Azide resistant mutants of *Azospirillum* on maize crop.

5.4.1 Plant growth parameters

Among the different growth parameters, significant increase in the plant height was noticed till end of growth period. Inoculation of Azide resistant mutants of *Azospirillum* showed increase in plant height of maize crop when compared to inoculated and uninoculated control. Increase in the height of crop plants due to inoculation of *Azospirillum* has been reported by several workers (Rangarajan *et al*., 1987; Subba Rao, 1993 and Hemavathi, 1997).

Plant growth promoting substances viz., IAA and GA play an important role in root elongation and shoot growth (Tien *et al*., 1979). *Azospirillum* enhances the IAA production which inturn enables the plants to grow better (Pathak *et al*., 1975 and Wang *et al*., 1995).

The result of the present investigation indicated in general, that the inoculation of Azide resistant mutants of *Azospirillum* with combination of N fertilizer showed increased shoot and root dry matter production of maize plant over controls at all stages of crop growth.

Similar reports were reported on maize crop by the mutant ACD-701 and ACD-802 over their wild types ACD-7 and ACD-8 respectively by Dayamani (2003). In pea plants which were inoculated with the Azi^R. *Rhizobium* showed higher shoot dry weight and N-uptake when compared to wild type inoculated plants (Ram *et al*., 1978 and Sharm *et al*., 1997).

5.4.2 Per cent nitrogen content in the plant

The per cent nitrogen content in plant, shoot and root increased significantly in the treatment inoculated with *Azospirillum* mutants. The results obtained were similar to studies made by Bhattarai and Hess (1993). Increased 'N' content of inoculated plants in the field at different stages during plant development have been observed in sorghum (Kapulnik *et al*., 1981; Sarig *et al*., 1986) and wheat (Boddey *et al*., 1986, Kapulnik, 1985; Zambre *et al*., 1984).

Boddey *et al.* (1986) have shown by ^{15}N fertilizer use that plants inoculated with live inoculation recovered more fertilizer N than the plants inoculated with autoclaved cells. In several experiments inoculation with N_2 fixation or 'N' assimilation measure (Vanberkum and Bohololol, 1980) N balance methods suggested significant amounts of N_2 fixation associated with grasses in some experiments.

Plant inoculated with *Azospirillum* were also shown the increased 'N' content in the seed. Similar results were also obtained in several experiments (Baldani *et al*., 1987; Kapulnik *et al*., 1984; Mertens and Hess, 1984). The possible mechanisms for higher 'N' accumulation may be the transfer of atmospheric nitrogen to the plant through bacterial nitrogen fixation (Lucky, 1988) and/or improved nitrogen uptake in the inoculated plants (Murty and Ladha, 1988).

According to Sharma *et al.* (1997) relationship between azide resistance and N-fixing ability in case of *R. loti* may be attributed to enhanced respiration and high level of cytochrome 0 and aa_2 under microaerobic culture condition has improved N-fixation activity.

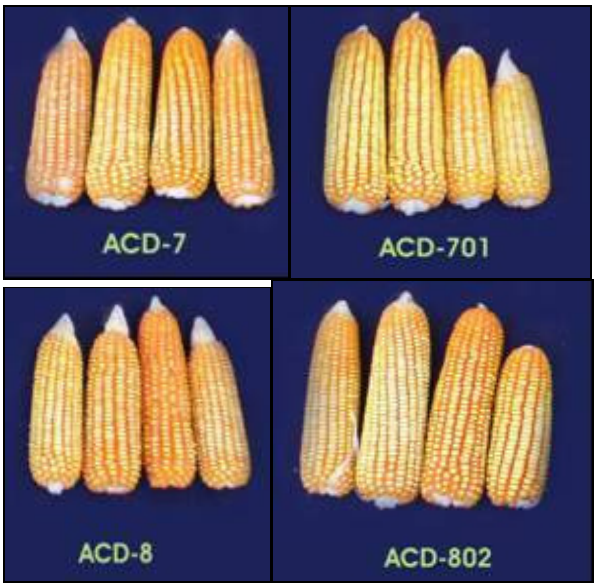

Plate.3: Growth of maize crop as influenced by Azide resistant mutant of Azospirillum

These results suggested that at least one of the mechanism may act for enhanced N-fixation by azide resistant mutants.

From the present investigation, it can be concluded that Azi^R mutants ACD-701 and ACD-802 can play an important role in enhancing the nitrogen availability to maize. These biofertilizers performed better in the presence of added N-fertilizers. Inoculation of *Azospirillum* strains in combination with 100 per cent N of RD can be used to get good yield in case of maize.

VI. SUMMARY

Increasing costs of chemical fertilizers, the environmental pollution caused by them and also the depletion of fossil fuel resources, raw materials for chemical fertilizer have called for more attention to the use of bioinoculants to supplement chemical fertilizers. Taking into considering the above factors, *Azospirillum* mutant strains with the enhanced nitrogen fixation efficiency were selected and screened.

The present investigation was undertaken to study the performance of *Azospirillum* mutants inoculated to the maize at two levels of nitrogen (100% and 75% of RD). Two higher azide resistance mutants of *Azospirillum* were selected and applied to the field condition to observe the performance of mutants on growth and yield parameters of maize crop. The results are summarized.

Results obtained from the field study revealed that, inoculation of azide resistant mutants of *Azospirillum* showed greater influence on plant growth parameter, endorhizosphere population, rhizosphere population, nutrient uptake and yield of maize as against inoculation of wild type *Azospirillum* reference strain and uninoculated control.

The maximum plant height, biomass and N uptake was observed in plants inoculated with ACD-701 and ACD-802, when compared to other treatments inoculated with wild type, reference strains and the uninoculated control.

The results revealed that *Azospirillum* mutants ACD-802 inoculation significantly increased the shoot length, root length, dry matter content of shoot and root and N uptake in maize plants over other inoculations and/or uninoculated controls. Application of 100% N of RD further enhanced the above plant growth parameters significantly over 75% N of RD.

Inoculation of *Azospirillum* mutants increased the colonization of *Azospirillum* in the rhizosphere and endorhizosphere. Increased population of *Azospirillum* was observed in the treatments inoculated with *Azospirillum* mutants along with nitrogenous fertilizers in the rhizosphere, compared with the treatments inoculated with wild type *Azospirillum* reference strains and uninoculated control. Maximum *Azospirillum* population was recorded in plants at 60 days after sowing.

The per cent nitrogen content in the plant shoot and root increased significantly in the treatments inoculated with the azide resistant mutants of *Azospirillum* compared to their respective wild types, reference strain and uninoculated control.

The chlorophyll content also increased gradually with the crop growth and there was significantly higher chlorophyll content in the treatments with the azide resistant mutants.

Application of azide resistant mutant of *Azospirillum* biofertilizer showed differential influence on grain N, cob length, cob weight, 100-test weight, grain yield and stover yield. The maximum yield was recorded in treatment ACD-802 + 100% RDN over wild types and control.

Among the two mutants the ACD-802 was found to be more competent than the ACD-701 and the wild types ACD-8, ACD-7. The mutants were found to be stable over a period of time and does not show reversion.

From the present investigation, it can be concluded that Azi^R mutants ACD-701 and ACD-802 can play an important role in the enhancing the nitrogen availability to maize. These biofertilizers performed better in presence of added N-fertilizers. Inoculation of *Azospirillum* strains in combination with 100 per cent N of RD can be used to get good yield in case of maize.

VII. REFERENCES

ABDEL-SALAM, M.S. AND KINGMULLER, W., 1987, Transposon Tn5 mutagenesis in *Azospirillum lipoferum*: Isolation of indole acetic acid mutants. *Molecular General Genetics*, **21**: 165-170.

ALBRECHT, S.L., OKON, Y., LONQUIST, J. AND BURRIS, R.H., 1982, Nitrogen fixation by corn *Azospirillum* associations in a temperate climate. *Crop Science*, **21**: 302-306.

ALEXANDRE, G., JACOUD, C., FAURE, D. AND BALLY, R., 1996, Population dynamics of a motile and a non-motile *Azospirillum lipoferum* strain during rice root colonization motility variation in the rhizosphere. *FEMS Microbiology,* **19** (4) : 271-278.

ANONYMOUS, 2003, *Economic Survey 2001-02*, Editors Government of India, Ministry of Finance and Economic Divisions, pp.15-20.

ARNON, D.I., 1940, Copper enzymes in isolated chloroplasts, 1-polyphinol oxidase in *Beta vulgaris. Plant Physiology*, **24** : 1-15.

ARSAC, J.F., LAMOTHE, C., MULARD, D. AND FAGES, J., 1990, Growth enhancement of maize (*Zea mays* L.) through *A. lipoferum* inoculation : effect of plant genotype and bacterial concentration. *Agronomie*, **10** (8) : 640-654.

BACA, B.E., SOTO URZUA, L., XOCHIHUA-CORONA, Y.G. AND CUERVO GARCIA, 1994, Characterization of two aromatic amino acids amino transferases and production of indole acetic acid in *Azospirillum* strains. *Soil Biology Biochemistry*, **26**: 57-63.

BALA G., AND GAUR, Y.D., 1997, Cultural, physiological and serological analysis of Azi^R mutants of *Bradyrhizobium* sp. (Cajanus). *Journal of Biochemistry and Biotechnology*, **6** : 111-118.

BALANDREAU, J., 1986, Ecological factors and adaptive processes in N_2 fixing bacterial populations of the plant environment. *Plant and Soil*, **90**: 73-92.

BALASUBRAMANIAM, A. AND KUMAR, K., 1989, Influence of *Azospirillum* biofertilizer on rice crop. *International Workshop and Symposium on Biological Nitrogen Fixation Associated with Rice Production*, CIRRI, Cuttack, December 28, 1988 to January 1, 1989.

BALDANI, J.E., CARUSO, L., HALDANI, V.L.D., GOI, S.R. AND DOBEREINER, J., 1997, Recent advances in BNF with non-legume plants. *Soil Biology and Biochemistry*, **29** : 911-922.

BALDANI, V.L.D. AND DOBEREINER, J., 1980, Host-plant specificity in the infection of cereals with *Azospirillum* sp. *Soil Biology and Biochemistry*, **12**: 86-90.

BALDANI, V.L.D., ALVAREZ, M.A., J.I., BALDANI, AND BALDANI, J.I., 1987, Inoculation of field grown wheat (*Triticum aestivum*) with *Azospirillum* spp. In Brazil. *Biological Fertility Soils*, **4** : 37-40.

BALDANI, V.L.D., BALDANI, J.L. AND DOBEREINER, J., 1983, Effects of *Azospirillum* inoculation on root infection and nitrogen incorporation in wheat. *Canadian Journal of Microbiology*, **29**: 924-929.

BAR, T. AND OKON, Y., 1993, Tryptophan conversion to indole-acetic acid via indole-3-acetamide in *Azospirillum brasilense* sp. 7. *Canadian Journal of Microbiology*, **38** : 81-86.

BARBER, L.E., TJEPKEMA, J.D., RUSSEL, S.A. AND EVANS, H.J., 1978, Acetylene reduction in the root environment of some grasses and other plants in Oregen. In: *Environmental Role of Nitrogen Fixing Blue-Green Algae and Asymbiotic Bacteria. Ecological Bulletin* (Stockholm), **26**: 366-372.

BARBIERI, P., BAGGIO, C., BAZZOCALUPO, M., GALLI, E., ZANETTI, G. AND NUTI, M.P., 1990, *Azospirillum* – Graminae interaction – Effect on indole – 3 Acetic acid. In: *Nitrogen fixation* Ed. Polsinelli, M., Materassi, R. and Veincenzini, M., Kluwer Academic, Dordrecht, pp.161-180.

BARBIERI, P., ZANELLI, T., GALLI, E. AND ZANETTI, G., 1986, Wheat inoculation with *Azospirillum brasilense* and some mutants altered in nitrogen fixation and indole-3 acetic acid production. *FEMS Microbiological Letters*, **36**: 87-90.

BASHAN, Y. AND HOLGUIN, G., 1995, Inter root movement of *Azospirillum brasilense* and subsequent root colonization of crop and weed seedlings growing in soil. *Microbial Ecology*, **29**: 269-281.

BASHAN, Y. AND LEVANONY, H., 1990, Current status of *Azospirillum* inoculation technology *Azospirillum* as a challenge for agriculture. *Canadian Journal of Microbiology*, **36**: 291-608.

BASHAN, Y., LEVANONY, H. AND ZIV-VECHT, O., 1987, The fate of field inoculated *Azospirillum brasilense* in wheat rhizosphere during the growing season. *Canadian Journal of Microbiology*, **35** : 691-697.

BASHAN, Y., REAM, Y., LEVANONY, H.L. AND SADE, A., 1989, Non specific response in plant growth yield and root colonization of non cereal crop plants to inoculation with *Azospirillum brasilense* cd. *Canadian Journal of Botany*, **67**: 1317-1324.

BATTARAI, T. AND HESS, D., 1993, Yield responses of Nepalese spring wheat (*Triticum aestivum* L.) cultivars to inoculation with *Azospirillum* spp. Of Nepalese origin. *Journal of Bacteriology*, pp. 3949-3955.

BEIJERINCK, M.W., 1922, *Azotobacter chroococcum* as indikator van de vruchtbarrheid van den grond ned akad wer verslsity gewone vergad afd natuurkd, **30**: 431-438.

BEIJERINCK, M.W., 1925, Uber ein *spirillum*, Welches freien stickstoff bidenkann. *Central Baktparasit Infect II Abstract Edition*, **63**: 353-359.

BELIMOV, A.A., KUNAKOVA, A.M., VASILYEVA, N.D., GRUZDEVA, E.V., VOROBIEV, N.I., KOJEMIAKOV, A.P., KHAMOVA, O.F., POSTAVSKAYA, S.M. AND SOKOVA, S.A., 1995, Relationship between survival rates of associative nitrogen-fixes on roots and yield response of plants to inoculation. *FEMS Microbiology Ecology*, **17** (3) : 187-197.

BERGE, O., FAGES, J., MULARD, D. AND BALANDREAU, J., 1990, Effect of inoculation with *Bacillus circulans* and *A. lipoferum* on crop yield in field grown maize. *Symbiosis Rehorot*, **9** (1-3) : 259-266.

BODDEY, R.M., BALDANI, V.C.D., BALDANI, J.I. AND DOBEREINER, J., 1986, Effect of inoculation of *Azospirillum* sp. on nitrogen accumulation by field grown wheat. *Plant and Soil*, **95** : 109-121.

BOTTINI, R., FULCHIERI, M., PEARCE, D. AND PHARIS, R.P., 1989, Identification of gibberellins Al, A and ISOA3 in cultures of *Azospirillum lipoferum*. *Plant Physiology*, **90**: 45-47.

BOUTON, J.H., SMITH, R.L., SCHANK, S.C., BOURTON, G.W, TYLER, M.E., LITTELL, R.C., GALLAHER, M.R. AND BERRY, K.H., 1979, Response of pearl millet inbreeds and hybrids to inoculation with *Azospirillum brasilense*. *Crop Science*, **19**: 12-16.

CASANOUAS, E.M., BARASSI, C.A., ANDRADE, F.H. AND SUELDO, R.J., 2003, *Azospirillum* inoculated maize plant responses to irrigation restraints imposed during flowering. *Cereal Research Communication*, **31** (3) : 395-402.

CASANOVAS, E.M., BARASSI, C.A. AND SUELDO, R.J., 2000, *Azospirillum* inoculation of maize seeds during imbibition. *Cereal Research Communications*, **28**: 25-32.

CHRISTIANSEN-WENIGER, C. AND VAN-VEEN, J.A., 1991, NH_4+ excreting *Azospirillum brasilense* mutants enhance the nitrogen supply of a wheat root. *Applied Environmental Microbiology*, **57**: 3006-3012.

COCHRAN, W.G., 1950, Estimation of bacterial density by means of the most probable number. *Biometrices*, **6**: 105-106.

CREUS, C.M., SUELDO, R.J. AND BARASSI, C.A., 2004, Water relations and yield in *Azospirillum* inoculated wheat exposed to drought in the field. *Canadian Journal of Botany*, **82** (2) : 273-281.

CROZIER, A., ARRUDA, P., JASMIN, J.M., MONTERIO, A.M. AND SANDBERG, G., 1988, Analysis of indole-3-acetic acid and related indoles in culture medium from *Azospirillum lipoferum* and *Azospirillum brasilense*. *Applied Environmental Microbiology*, **54**: 2833-2837.

DAYAMANI, 2003, Characterisation of azide resistant mutants of *Azospirillum*spp. *M.Sc. (Agri.) Thesis*, University of Agricultural Sciences, Dharwad.

DE-GALLO, M.M., GRATANI, L. AND MARPURGO, 1982, Mutation in *Azospirillum brasilense*. In: *Azospirillum* III. Genetics, Physiology Ecology, Springer Verlag Publications, Berlin, Heideburg, New York.

DEVI, L.G., 2002, Forage yield of maize (*Zea mays* L.) as influenced by nitrogen levels and biofertilizers. *Forage Research*, **27** (4) : 263-266.

DOBEREINER, J., 1977, Physiological aspects of nitrogen fixation in grass bacteria association. In: *Recent Developments in Nitrogen Fixation*, Ed. Newton, W.E., Postgate, J.R. and Rodriguez-Barrueco, C. Academic Press, London, pp.513-522.

DOBEREINER, J. AND BALDANI, V.L.D., 1979, Selective infection of maize roots by streptomycin-resistant *Azospirillum lipoferum* and bacteria. *Canadian Journal of Microbiology*, **25**: 1264-1269.

DOBEREINER, J. AND BALDANI, V.L.D., 1981, Prospects for inoculation of grasses in the *Azospirillum* spp. In: *Associative N_2 fixation Vol.II*, Ed. Vose, P.B., Ruschel, A.P. CRC Press, Palm Beach, II, Untied States of America, pp.1-9.

DOBEREINER, J. AND BODDEY, R.M., 1980, Nitrogen fixation in association with graminae, In: *Proceedings of Fourth International Symposium Nitrogen Fixation*, Canberra, Ed. Gibson, A.H., Australian Academy of Sciences.

DOBEREINER, J. AND DAY, J.M., 1976, First international symposium on nitrogen fixation. In: *Proceedings of International Symposium on Nitrogen Fixation*, Ed. Newton, W.E. and Nyman, C.J., Washington State University Press, Pullman, W.A., pp.518-538.

DOBEREINER, J. AND DEPOLLI, H., 1980, Diazotrophic rhizocoenesis. In: *Nitrogen Fixation*, Ed. Steward, W.D.P. and Gallon, J.R. Academic Press, pp.301-333.

DOBEREINER, J., MARRIEL, I.E. AND NERY, M., 1976, Ecological distribution of *Sprillum lipoferum* (Beijerinck). *Canadian Journal of Microbiology*, **22**: 1464.

FALLIK, E. AND OKON, Y., 1996, Inoculates of *Azospirillum brasilense*, Biomass production. Survival and growth promotion of *Seteria italica* and *Zea mays*. *Soil Biology and Biochemistry*, **28**: 123-126.

FALLIK, E., OKON, Y., EPISTIEN, E., GOLDMAN, A. AND FISCHER, M., 1989, Identification and quantification of IAA and IBA in *Azospirillum brasilense* – inoculated maize roots. *Soil Biology and Biochemistry*, **21**: 147-153.

FRANCHE, C. AND ELMERICH, C., 1981, Physiological properties and plasmid content of several strains of *Azospirillum brasilense* and *A. lipoferum*. *Annual Microbiology*, **123**: 3-18.

GADAGI, R., 1999, Studies on *Azospirillum* isolates of ornamental plants and their effect of *Gailardia puchella* var. *picta Fouger*. *Ph.D. Thesis*, University of Agricultural Sciences, Dharwad.

GANGULY, T.K. AND MANNA, M.C., 1999, *Azospirillum brasilense* – An associative organism in Agriculture – A review. *Agriculture Reviews*, **20** (3/4) : 220-228.

GASKINS, M.H. AND HUBBEL, R.H., 1979, Response of non leguminous plants to root inoculation with free-living diazotrophic bacteria. In: *The Soil Root Interface*, Ed. Harley, J.L. and Russel, R.S., Academic Press, Inc, New York, pp.175-182.

GERK, L.P., GILCHRIST, K. AND KENNEDY, I.R., 2000, Mutants with enhanced nitrogenase activity in hydroponic *Azospirillum brasilense* wheat associations. *Applied and Environmental Microbiology*, **66** (5) : 2175-2184.

GOMEZ, K.A. AND GOMEZ, A.A., 1984, *Statistical Procedures for Agricultural Research*. An International Rice Research Institute Book, Wiley-Inter Science Publication, New York, United States of America.

GORDEN, S.A. AND PALEG, L.G., 1957, Quantitative measurement of Indole Acetic Acid. *Plant Physiology*, **10**: 37-48.

GOVINDAN, M. AND PURUSHOTHAMAN, D., 1985, Association of nitrogen fixing bacteria with certain plantation crops. *National Academy of Science Letters*, **8**: 163-165.

GOVINDAN, M., 1993, *Azospirillum* associated with rice in acidic soils of coastal Karnataka and its possible use as a bioinoculatn. *Ph.D. Thesis*, University of Agricultural Sciences, Bangalore.

HARTMANN, A. AND BURRIS, R.H., 1987, Regulation of nitorgenase activity by oxygen in *Azospirillum brasilense* and *Azospirillum lipoferum*. *Journal of Bacteriology*, **169**: 944-948.

HARTMANN, A., FU, H. AND BURRIS, R.H., 1988, Influence of amino acids on nitrogen fixation ability and growth of *Azospirillum* spp. *Applied and Environmental Microbiology*, **54**: 87-93.

HARTMANN, A., PRABHU, S.R. AND GALINSK, E.A., 1991, Osmotolerance of diazotrophic rhizosphere bacteria. *Plant and Soil*, **137** : 105-109.

HARTMANN, A., SINGH, M. AND KLINGMULLER, W., 1983, Isolation and characterization of *Azospirillum* mutants excreting high amounts of indole acetic acid. *Canadian Journal of Microbiology*, **29**: 916-920.

HAYES, J.D. AND WOLF, C.R., 1990, Molecular mechanisms of drug resistance. *Biochemical Journal*, **272**: 281-295.

HEGAZI, N.A., KHAWAS, H. AND MONIB, M., 1981, Inoculation of wheat *Azospirillum* under Egyptian conditions. In: *Current perspectives in nitrogen fixation*. Ed. Gibson, A.H. and Newton, W.E., *Proceedings of 4th International Symposia on Nitrogen Fixation*, Canberra, p.493.

HEMAVATHI, M., 1997, Effect of organic manures and biofertilizer on growth and productivity of chrysanthemum (*Chrysanthemum morifolium* Ramat) Cv. Local Yellow, *M.Sc.(Agri.) Thesis*, University of Agricultural Sciences, Bangalore.

HENLIN, T., GUCKERT, A. AND BALANDREAU, J., 1987, Stimulation of root exudation of rice seedlings of *Azospirillum* strains: Carbon budget under gnotobiotic conditions. *Biology and Fertility of Soils*, **4**: 9-144.

HERNANDEZ, Y., SOGO, J. AND SARMIENTO, M., 1997, *Azospirillum* inoculation on *Zea mays*. *Cuban Journal of Agricultural Science*, **31** (2) : 203-209.

HOREMANS, S., DE KONINCK, K., NEURAY, J., HERMANS, R. AND KRLASSAK, 1986, Production of plant growth substances by *Azospirillum* sp. and other rhizosphere bacteria. *Symbiosis*, **2**: 341-346.

HUMPHRIES, E.C., 1956, Mineral components and ash analysis, In: *Modern Methods of Plant Analysis*, Ed. Peach, K. and Tracey, M.V., Springer, Verlag, Berlin, pp.468-502.

INBAL, E. AND FELDMAN, M., 1992, The response of a hormonal mutant of common wheat to bacteria of the genus *Azospirillum*. *Israel Journal of Botany*, **31**: 257-263.

JACKSON, M.L., 1973, *Soil Chemical Analysis*, prentice Hall of India Private Limited, New Delhi, p. 498.

JAIN, D.K. AND PATRIQUIN, D.G., 1984, Root hair deformations bacterial attachment and plant growth in wheat *Azospirillum* association. *Applied and Environmental Microbiology*, **48**: 1208-1213.

JAIN, D.K. AND PATRIQUIN, D.G., 1985, Characterization of a substance produced by *Azospirillum* which causes branching of wheat root hairs. *Canadian Journal of Microbiology*, **31**: 206-210.

KAPLUNIK, Y., KIGET, J., OKON, Y., NUR, I. AND HENIS, Y., 1981, Effect of *Azospirillum* inoculation on some growth parameters and N-content of wheat, sorghum and panicum. *Plant and Soil*, **61**: 65-70.

KAPLUNIK, Y., OKON, Y. AND HENIS, Y., 1985, Changes in root morphology of wheat caused by *Azospirillum* inoculation. *Canadian Journal of Microbiology*, **31**: 881-887.

KAPULNIK, Y., GAFNY, R. AND OKON, Y., 1984, Effect of *Azospirillum* spp. Inoculation on root development and NO_3 uptake in wheat (*Triticum aestivum* Miriram) in hydroponic systems. *Canadian Journal of Botany*, **63** : 627-633.

KHAMMAS, K.M., AGERON, E., GRIMONT, P.A.D. AND KAISER, P., 1989, *Azospirillum irakensse* sp. Nov., a nitrogen fixing bacterium associated with rice roots and rhizosphere soil. *Research Microbiology*, **140**: 679-681.

KOLB, W. AND MARTIN, P., 1985, Response of plant roots to inoculation with *Azospirilum brasilense* and to application of indole-acetic acid. In: *Azospirillum III. Genetics, Physiology, Ecology*, Ed. Klingumuller, W. Springer-Verlag, Berlin, pp.215-221.

KREIG, N.R. AND DOBEREINER, J., 1984, Genus *Azospirillum*, Tarrand, Kreing and Dobereiner, 1979, (Effective Publication: Tarrand, Kreing and Dobereiner, 1978, 948). In: *Bergey's Manual of Systematic Bacteriology*, 9th Ed. Williams, V.I. and Wlkins, Baltimore, pp.94-104.

KRISHNARAJ, P.U., 1996, Genetic characterization of mineral phosphate solubilization in *Pseudomonas* sp. *Ph.D. Thesis*, Indian Agricultural Research Institute, New Delhi.

KUMAR, S., PARODA, S. AND DADARWAL, K.R., 1995, Root growth stimulating *Azospirilla* associated with roots of sunflower (*Helianthus annuus* L.). *Indian Journal of Experimental Biology*, **38**(8): 565-570.

LAM, S.T., ELLIS, D.M. AND LIGON, J.M., 1990, Genetic approaches for studying rhizosphere colonization. *Plant and Soil*, **129** : 11-18.

LAXMIKUMARI, M., KAVIMANDAN, S.K. AND SUBBA RAO, N.S., 1976, Occurrence of nitrogen fixing *Spirillum* in roots of rice, sorghum, maize and other plants. *Indian Journal of Experimental Biology*, **9**: 638-639.

LINNETT, P.E. AND BEECHEY, P.D., 1979, Inhibitors of the ATP synthetase system. *Methods in Enzymology*, **55**: 472-518.

LUCKY, R.M.N., 1988, Plant growth altering effects of *Azospirillum brasilense* and *Bacillus* C-11-25 on two wheat cultivators. *Journal of Applied Bacteriology*, **64** (3) : 187-196.

MACHADO, H.B., FUNAYAMA, S., RIGO, L.V. AND REDROSA, F.O., 1990, Excretion of ammonium by *Azospirillum brasilense* mutants resistant to ethylenediamine. *Canadian Journal of Microbiology*, **57**: 549-553.

MAGALHAES, F.M.M., BALDANI, J.I., SOUTO, S.M., KUYKENDALL, J.R. AND DOBERINER, J., 1983, A new acid tolerant *Azospirillum* species. *Annals of Academic Bras Cien.*, **55**: 417-420.

MAHESHKUMAR, K.S., 1997, Studies on microbial diversity and their activity in soil under bamboo plantation. *M.Sc.(Agri.) Thesis*, University of Agricultural Sciences, Dharwad.

MARTENS, T. AND HESS, D., 1984, Yield increase in spring wheat (*Triticum aestivum* L.) inoculated with *Azospirillum lipoferum* under green house and field conditions of temperate origin. *Plant and Soil*, **82** : 87-99.

MERINA, P.S., 1991, Response of certain horticultural crops to inoculation with fungi. *Azospirillum* and *Phosphobacteria*. *M.Sc.(Agri.) Thesis*, Tamil Nadu Agricultural University, Coimbatore.

MICHIELS, K., DE TROCH, P., ONYOCHA, I., VANGASE, ELMERICH, C. AND VARDERLEYDEN, J., 1989, Plasmid isolation and mapping of two *Azospirillum brasilense* loci that affect polysaccharide synthesis. *Plasmid*, **21**: 142-146.

MORANDINI, P., VIGNOLA, R., VITTROIOSO, P. AND BENSICHEM, M.L.V., 1990, Efficiency of *Azospirillum* inocula in peat formation maize (*Zea mays*) root colonization and effect on initial plant growth.

MORI, E., FANI, R., GALLORI, E., FANTAPPLE, O., BASSICALUPO, M., 1992, Mutants of *Azospirillum* altered in the uptake of iron. *Symbiosis Rehovot*, **13**: 155-122.

MURTY, M.G. AND LADHA, J.K., 1988, Influence of *Azospirillum* inoculation on the mineral uptake and growth of rice under hydroponic conditions. *Plant and Soil*, **108**: 281-285.

OKON, Y. AND HADAR, Y., 1987, Microbial inoculants as crop yield enhancers. *CRC Critical Reviews in Biotechnology*, **6**: 61-85.

OKON, Y., ALBRECHT, S.L. AND BURRIS, R.H., 1976, Factors affecting growth and nitrogen fixation of *Sprilum lipoferum*. *Journal of Bacteriology*, **127**: 1248-1254.

OKON, Y., ALBRECHT, S.L. AND BURRIS, R.H., 1977, Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. *Applied and Environmental Microbiology*, **33**: 83-85.

OKON, Y., KAPULNIK, Y., SARIG, S., NUR, I., KIEGEL, J. AND HENSI, Y., 1981, *Azospirillum* increases cereal crop yields in fields of Israel. In: *Current Perspectives in Nitrogen Fixation. Proceedings of 4th International Symposium on Nitrogen Fixation*, Ed. Gibson, A.H. and Newton, W.E., Canberra, p.492.

OMAY, S.H., SCHMIDT, W.A. AND MARTIN, P., 1993, Indole acetic acid production b the rhizosphere bacterium *Azospirillum brasilense* cd under *in vitro* conditions. *Canadian Journal of Microbiology*, **39**: 187-192.

PALEG, L.G., 1965, Physiological effects of gibberllins. *Annual Review of Plant Physiology*, **16**: 291-322.

PAREDES, C.E., CARCANO, M.M., MASCARUA, E.M.A. AND CABALLERO, M.J., 1988, Response of maize to inoculation with *A. brasilense. Revista Latimoamericana de Microbiologia*, **30** (4) : 351-355.

PATHAK, A.N., TIWARI, K.N. AND SINGH, K., 1975, Zinc-phosphate inter relationship in rice. *Journal of Indian Society of Soil Science*, **23** : 477-483.

PATIL, S.J., WALI, M.C., HARLAPUR, S.I AND PRASHANT, M., 2000, *Maize research in Northern Karnataka*, University of Agricultural Sciences, Dharwad.

PATRIQUIN, D.G. AND DOBEREINER, J., 1978, Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. *Canadian Journal of Microbiology*, **24**: 734-747.

PATRIQUIN, D.G., DOBEREINER, J. AND JAIN, D.K., 1983, Sites and processes of association between diazotrphos and grasses. *Canadian Journal of Microbiology*, **29**: 900-915.

PEDROSA, F.O., DOBEREINER, J. AND YATES, M.G., 1980, H_2 dependent growth and autotrophic CO_2 fixation by *Derxia*. *Journal of General Microbiology*, **19**: 547-551.

PEREIRA, J.A.R. 1990, Inoculation of maize and sorghum with nitrogen fixing bacteria in the field. *Documentos Empresa Capixaba de Pesquisa Agropecuaria*, **65** : 128.

PIPER, C.S., 1966, *Soil and Plant Analysis*, Academic Press, New York, p.366.

PRATHIBHA C.K., 1993, Interactions of *Azospirillum* sp. and *Pseudomonas striata* or *Glomus fasciculatum* on growth, yield and nutrient uptake of hybrid cotton. *M.Sc.(Agri.) Thesis*, University of Agricultural Sciences, Dharwad.

PURUSHOTHAMAN, D., JAYANATH, M.K. AND GUNASHEKARAN, S. 1988, Association of *Azospirillum* with the roots of upland rice. In: *Biofertilizers Potentialities and Problems*, Ed. Sen, S.P. and Patil, P. *Plant Physiology Forum*, Calcutta, Naya Prakash, Calcutta.

QUINTERO, M.J. AND GARZA, R., 1978, Isolation of *Spirillum lipoferum* from tropical grasses in Mexico. In: *Limitations and Potentials of Biological Nitrogen Fixation in the Tropics*. Ed. Dobereiner, J. *et al*., Plenum Press, New York.

RAI, R., 1985, Studies on nitrogen fixation by antibiotic resistant mutants *Azospirillum brasilense* and their interaction with cheena (*Panicum miliaceum* L.) genotypes in calcareous. *Journal of Agricultural Science*, Camb, **105**: 261-270.

RAM, J., GROVER, R.P., REWORI, R.B. AND KUMAR, S., 1978, Improvement in the nitrogen fixing effectiveness of *R. leguminosorum* by incorporating genetic resistance to azide. *Indian Journal of Experimental Biology*, **16**: 1321-1322.

RANGARAJAN, M., KANDASAMY, D., NARAYANA, R., PONNIAH, C., GOVINDARAJAN, K. AND OBLISAMI, G., 1987, Exploitation of microbial system associated with plant species for the eco-development of western ghats region. *Final Technical Report of D.O.E. Project*, p. 38.

RANGASWAMI, G. AND BAGYARAJ, D.F., 1996, Agricultural Microbiology, Asian Publishing House, New Delhi.

RAO, A.V. AND VENKATESHWARALU, B., 1982, Associative symbiosis of *Azospirillum lipoferum* with dicotyledanous, succulent plants, the Indian desert. *Canadian Journal of Microbiology*, **28**: 778-782.

REINHOLD, B., HUREK, T., FENDIRK, I., POT, B., GILLIS, M., KERSTER, K., THIELMANS, S. AND DELY, J., 1987, *Azospirillum halopraferans* s. Nov. a nitrogen – fixing organism associated with roots of kallar grass (*Leptchola fusca* L.) Kunth. *International Journal of Systemic Bacteriology*, **37**: 43-51.

REYNDERS, L. AND VLASSAK, K., 1979, Conversion of tryptophan to indole acetic acid by *Azospirillum* sp. *Soil Biology and Biochemistry*, **11**: 345-348.

REYNDERS, L. AND VLASSAK, K., 1982, Use of *Azospirillum brasilense* as biofertilizer in intensive wheat cropping. *Plant and Soil*, **66**: 217-227.

RIBANDO, C.M., PACCUSSE, A.N., RONDANINI, D.P. AND CURA, J.A., 1998, *Azospirillum* – maize association : Effects on dry mater yield and nitrate reductase activity. *Agricultura Tropica et Subtropica*, **31** : 61-70.

RODRIGUEZ-CACERES, E.A., 1982, Improved medium for isolation of *Azospirillum* spp. *Applied Environmental Microbiology*, **44** : 990-991.

RUSCHEL, A.P. AND VOSE, P.R., 1981, Discussion on terminology. In: *Association nitrogen fixation*. Ed. Vose. P.R. and Ruschel, A.P., CRC Press, Boca Raton, Florida, **1**: 241.

SANTHANAKRISHNAN, P. AND OBLISAMI, G., 1988, Effect of the bacterial inoculants and growth regulator on the growth of mulberry cuttings. *Proceedings on Biofertilizers*, Tamil Nadu Agricultural University, Coimbatore, pp.93-95.

SARIG, S., BLUM, A. AND OKON, Y., 1988, Improvement of the water and yield of field-grown grain sorghum (*Sorghum bicolor*) by inoculation with *Azospirillum brasilense*. *Journal of Agricultural Sciences*, **110**: 271-277.

SCHANK, S.C., SMITH, R.H., WEISER, G.C., ZUESER, D.A., BONTAIN, J.H., QUENSEN BERRY, K.H., TYLER, M.E., MILAM, J.L. AND HITTELL, R.C., 1979, Florescent antibody technique to identify *Azospirillum brasilense* amino acids with roots of grasses. *Soil Biology and Biochemistry*, **11** : 287-295.

SESSITSCH, A., JIEMBA, P.K., HARDARSON, G., AKKERMANS, A.D.L. AND WILSON, K.J., 1996, Measurement of the competitiveness index of Rhizobium tropics. Strain CIAT899 derivatives marked with the gus A gene. **29** (7) : 1099-1110.

SHARMA, C.R., ACHARYA, A. AND GUPTA, M.K., 1997, Effect of *Azospirillum* inoculation on production of rice crop in mid hill zone of Himachal Pradesh. In: *Association of Microbiologists of India, 33rd Annual Conference*, Nov, 5-7, held at Goa University, Goa (Abstract) p.34.

SHUBHA, S., 1999, Characterization of ethylene diamine resistant mutants of *Azospirillum* spp. derived from wheat. *M.Sc.(Agri.) Thesis*, University of Agricultural Sciences, Dharwad.

SINGH, M. AND WENZEL, W., 1982, Detection and characterization of plasmids in *Azospirillum*. In: *Azospirillum Genetics, Physiology, Ecology*, Ed. Klingmuller, W., Birkhauser, Basel, pp.44-51.

SOMESEGARAN, P. AND HOBEN, H.J., 1994, *Hand book of Rhizobia: Methods in Legume-Rhizobium Technology*, (ed.) P. Somasegaran and H.J. Hoben, Spring-Verlag, New York, pp.31-38.

STANCHEVA, I. AND DINEV, N., 1992, Effects of inoculation of maize and species of tribe triticaceae with *Azospirillum brasilense. Journal of Plant Physiology*, **140** (5) : 50-552.

STANCHEVA, I., DIMITORV, I., KALOYANOVA, N. AND ANGELOV, M., 1992, Effects of inoculation with *Azospirillum brasilense* on photosynthetic enzyme activities and grain yield of maize. *Agronomie*, **12** (4) : 319-324.

STANCHEVA, I., KALOYANOVA, N., LYUBENOVA, Z. AND ANGELOV, M., 1991, Effect of inoculation with *Azospirillum brasilense* on conditions. *Fiziologiya na Rasteniyata*, **17** (3) : 3-8.

SUBBA RAO, N.S., 1981, Response of crops to *Azospirillum* inoculation in India. In: *Associative Nitrogen Fixation*, Vol.I. Ed. Vose, P.B. and Ruschel, A.P. CRC Press, Baco Raton, Florida, pp.137-144.

SUBBA RAO, N.S., 1986, Cereal nitrogen fixation research under the BNF coordinated project of the ICAR. In: *Cereal Nitrogen Fixation*, Ed. S.P. Wani, International Crop Research Institute Semi Arid Tropics. Patancheru, India, pp.23-30.

SUBBA RAO, N.S., 1993, Interaction of nodulated tree species with other microorganisms and plants. In: *Symbiosis in Nitrogen Fixing Trees*, Ed. Subba Rao, N.S. and Rodriguez-Barrueco, C., Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, p.250.

SUBBIAH, B. V. AND ASIJA, G. L., 1956, A rapid procedure for the estimation of available nitrogen in soils. *Current Science*, **25** : 259-260.

SUMNER, M.E., 1990, Crop responses to *Azospirillum* inoculation. *Advances in Soil Sciences*, **12**: 54-84.

SWEDRZNSKA, D. AND SAWICKA, A., 2001, Effect of inoculation on population number of *Azospirillum* bacteria under winter wheat, oat and maize. *Polish Journal of Environmental Studies*, **10** (1) : 21-25.

TAMILVENDAN, K. AND PURUSHOTHAMAN, D., 1996, Developing phosphorus solubilizing strains in *Azospirillum*. *Proceedings of the National Seminar on Microorganisms in Sustainable Agriculture*, Madurai, pp.79-89.

TARRAND, J.J., KRIEG, N.R. AND DOBERINER, J., 1978, A taxonomic study of the *Spirillum lipoferum* group, with descriptions of new genus. *Azospirillum* gen. Nov. and two species. *Azospirillum lipoferum* (Beijerinek) comb, Nov. and *Azospirillum brasilense* sp. nov. *Canadian Journal of Microbiology*, **24**: 967-980.

TIEN, T.M., GASKINS, M.H. AND HUBBELL, D.H., 1979, Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum*). *Applied and Environmental Microbiology*, **37**: 1016-1024.

TILAK, K.V.B.R., SINGH, C.S. AND RANA, J.P.S., 1981, Effects of combined inoculation of *Azospirillum brasilense* with *Rhizobium trifoli*, *Rhizobium meliloti* and *Rhizobium* sp. (cowpea miscellary) on nodulation and yield of clover (*Trifolium repense*), Lucerne (*Medicago sativa*) and chickpea (*Cicer arietinum*). *Zentralblatt fur Baketeriologie* II. Abt. **136**: 117-120.

TYLER, M.E., MILAM, J.R., SMITH, R.L., SCHANK, S.C. AND SUBERER, D.A., 1979, Isolation of *Azospirillum* from diverse geographic regions. *Canadian Journal of Microbiology*, **25**: 693-697.

UMALI-GRACIA, M., HUBBELL, D.H., GASKINS, M.H. AND GAZZO, F.B., 1981, Association of *Azospirillum* with grass roots. *Applied and Environmental Microbiology*, **29**: 219-226.

VAN DOMMELEN, A., VAN BOSTELAERE, E., KEIJERS, V. AND VENDERLEYDEN, J., 1997, Genetics of *Azospirillum brasilense* with respect of ammonium transport, Sugar uptake and chemotoxis. *Plant and Soil*, **4**: 156-160.

VANBEVKUM, P. AND BOHLOOL, B.B., 1980, Evaluation for nitrogen fixation by bacteria in association with roots of tropical grasses. *Microbiological Reviews*, **44** : 491-517.

VASUDEVA, M., KHARB, P., VASHISHT R.K., NARULA, N. AND MERBACH, W., 2003, Azide resistance in *Azotobacter chroccum* and its relationship with respiratory activity, ATP concentration, cytochrome C-oxidase and nitrogen fixation. *Indian Journal of Microbiology*, **49**: 51.

VEENA, S.C., 1999, Development of inoculum consortia for enhanced growth and nutrient uptake by sorghum. *M.Sc.(Agri.) Thesis*, University of Agricultural Sciences, Dharwad.

WANG, X., ZHANG, S.Z., MUNG, S.J. AND ZHANG, 1995, The effect of joint application of phosphorus and zinc in increasing the yield of summer soybean. *Henan Nangye Kenue*, **1** : 20-22.

WANI, S.P., 1992, *Role of Biofertilizers in Upland Crop Production*, Semi Arid Tropics (ICRISAT), pp.91-112.

WENIGER, C. AND VANDERLEYDEN, J., 1994, Ammonium excreting *Azospirillum* sp. become intercellularly established in maize (*Zea mays*) paranodules. *Biology and Fertility of Soils*, **17**: 1-8.

WENIGER, C., 1992, N_2-fixation by ammonium excreting *Azospirillum brasilense* in auxins – induced root tumours of wheat (*Triticum aestivum* L.). *Biology and Fertility of Soils*, **13**: 165-172.

WENIGER, C., 1997, Ammonium excreting *Azospirillum brasilense* C_3 – *gus* A inhabiting induced tumors along stem and roots of rice. *Soil Biology and Biochemistry*, **29**: 943-950.

WOODARD, H.J. AND BLY, A., 2000, Maize growth and yield response to seed inoculated N_2 fixing bacteria under dry land production conditions. *Journal of Plant Nutrition*, **23**: 55-65.

YADAV, A.S., SHAIKH, A.A., SHINDE, A.B. AND HARINARAYANA, G., 1989, Effects of growth hormones, biofertilizer and micronurients on the yield of pearl millet. *Journal of Maharashtra Agricultural Universities*, **15** (2) : 159-161.

ZAADY, E. AND OKON, Y., 1990, Cultural condition affecting *A. brasilense* cell aggregation and absorption to maize roots. *Soil Biology and Biochemistry*, **22** (8) : 1103-1107.

ZAMBRE, M.A., KONDE, B.K. AND SONAR, K.R., 1984, Effect of *Azotobacter chrococcum* and *Azospirillum brasilense* inoculation under graded levels of nitrogen on growth and yield of wheat. *Plant and Soil*, **79** : 61-67.

ZIMMER, W. AND BOTHE, W., 1988, The phytohormonal interaction between *Azospirillum* and wheat. *Plant and Soil*, **110**: 239-247.

ZIMMER, W., APARICO, C., EMERICH, C.E., 1991, Relationship between tryptophan biosyntehsis and indole-3-acetic acid production in *Azospirillum*: Identification and sequencing of a trp GDC cluster. *Molecular and General Genetics*, **229**: 41-51.

APPENDIX I

Chemical Composition of Media Used

N-free semi-solid malate medium (Okon *et al.*, 1977)	
Malic acid	5.0 g
KOH/NaOH	3.0 g
K_2HPO_4	0.5 g
$FeSO_4 . 7H_2O$	0.05 g
$MnSO_4$	0.01 g
$MgSO_4 . 7H_2O$	0.1 g
NaCl	0.02 g
$NaCl_4 . 2H_2O$	0.01 g
$NaMoO_4$	0.002 g
Agar	3.0 g
Distilled water	1000 ml
pH	6.6 to 7.0
BTB (0.5% alcohol solution)	2 ml
Potato infusion agar (Okoh *et al.*, 1977)	
Sucrose	2.5 g
Malic acid	2.5 g
Trace element solution	2 ml
Vitamin solution	1.0 ml
Potato	2000 g
pH	7.0
Agar	15.0 g
Distilled water	1000 ml
M-9 malate (Mg-M) medium	
Sterile double distilled water	750 ml
5 x Mg salts	200 ml
Glucose (20% solution)	20 ml
5 x mg salts	
$NaHPO_4 . 2H_2O$	38.3
KH_2PO_4	15.0
NaCl	2.5
NH_4Cl	5.0
To make solid MG M medium, Difco agar was added at the rate of 15 g/litre	

Congo red medium for purity checks (Rodriguez Caceres, 1982)	
K_2HPO_4	0.5 g
$MgSO_4$. $7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
$FeCl_3$. 6 H_2O	0.015 g
Malic acid	5.0 g
KOH	4.8 g
2 Agar	20.0 g
Congored (1:4000 aqueous solution)	15.0 ml
Distilled water	1000 ml
Luria agar	
Tryptone	5.0 g
Yeast extract	3.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.0
Pikovaskaya's medium	
Glucose	10.0 g
TCP	5.0 g
NH_4SO_4	0.5 g
KCl	0.2 g
$MgSO_4$	0.1 g
$MnSO_4$	Trace
Distilled water	1000 ml
MPSS broth (g/litre)	
Peptone (difco)	5.0
Succinic acid (free acid)	1.0
$(NH_4)_2SO_4$	1.0
$MgSO_4$. $7H_2O$	1.0
$FeCl_3$. $6H_2O$	0.002
$MnSO_4$. H_2O	0.002
pH	7.0

EVALUATION OF AZIDE RESISTANT MUTANTS OF *AZOSPIRILLUM* ON MAIZE

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ABSTRACT

An investigation was carried out to evaluate Azide resistant mutants of *Azospirillum* on maize at two levels of nitrogen (100% and 75% of RD). Two higher azide resistant mutants of *Azospirillum* were selected and applied to the field to observe the performance of mutants on growth and yield parameters of maize crop.

Results obtained from the field study revealed that, inoculation of azide resistant mutants of *Azospirillum* showed greater influence on plant growth parameter, endorhizosphere and rhizosphere population, nutrient uptake and yield of maize as against inoculation of wild type *Azospirillum* reference strain and uninoculated control. The maximum plant height, biomass and N uptake were recorded in plants inoculated with ACD-701 and ACD-802 compared to other treatments inoculated with wild type, reference strains and the uninoculated control.

Azospirillum mutants, ACD-802 inoculation significantly increased the shoot length, root length, dry matter content of shoot and root and N uptake in maize plants over other inoculations and/or uninoculated control. Application of 100% RDN further enhanced the above plant growth parameters significantly over 75% RDN. Inoculation of *Azospirillum* mutants increased the colonization of *Azospirillum* in the rhizosphere and endorhizosphere. Increased population of *Azospirillum* was observed in the treatments inoculated with *Azospirillum* mutants along with nitrogenous fertilizers in the rhizosphere, compared to treatments inoculated with wild type *Azospirillum* reference strains and uninoculated control. Maximum *Azospirillum* population was recorded in plants at 60 days after sowing. It can be concluded that Azi^R mutants ACD-701 and ACD-802 can play an important role in enhancing the nitrogen availability to maize. These biofertilizers performed better in the presence of added N-fertilizers. Inoculation of *Azospirillum* strains in combination with 100 per cent RDN can be used to get good yield in case of maize.