2. REVIEW OF LITERATURE

2.1. Significance of tomato

Tomato is herbaceous, usually sprawling plant in the Solanaceae or nightshade family, grown widely for its edible fruits. China is the leading producer with 32,540,040 metric ton (FAO, 2008). United states, the leading importer of fresh tomato received 25 per cent of the global value and volume. Indian annual population growth rate exceeds its food production, being 2.7 and 2.9 respectively between 1990 and 1997, which narrowed to 2.6 and 2.7 between 1998 and 2003 (FAO, 2006) necessitating food importation. Total tomato production in India varies between 889000 and 898000 from year 2004 and 2013 (FAO, 2013). The average tomato yield in India is 10 t ha$^{-1}$, which was lower than 13.5 t ha$^{-1}$ average yield in tropical Asia and world average of 22 t ha$^{-1}$ (FAO, 2013).

Although studies conducted in the tropics showed significant increase in nutrient status and yield of tomato due to application of inorganic fertilizers, high cost and scarcity of inorganic fertilizer pose constraints to its use especially among small-scale farmers in Africa (Ogballu, 1999).

It is one of the widely grown and consumed vegetable crops in the world. India’s annual produce of tomato accounts for nearly, 7.1 million tones and among the states, Tamil Nadu stands seventh in tomato production. The production of tomato is greatly influenced by multiple factors including but not limited to the cultivars, type of soil, fertilizers used. The nutrients such as N, P, K, Mg and Ca are required by tomato crops at the right time and in right quantity for good production and yield (Olaniyi and Ajibola, 2008).
2.2. *Azospirillum*

The genus *Azospirillum* comprises free living nitrogen fixing rhizosphere bacteria belonging to α -subclass of Proteobacteria. The first species of this genus, originally named *Spirillum lipoforum*, was isolated by Beijerinck (1925). Forgotten for half a century, *Azospirillum* was rediscovered in 1970’s and gained reputation of being the most studied bacterium. Dobereiner and Day (1976) reported that the nitrogen fixing *Azospirillum* was a very common root and soil inhabitant.

There are currently 7 species within the genus *Azospirillum*. *Azospirillum brasilense*, *A. lipoforum*, *A. amazonense*, *A. halopraeferens* and *A. irakense* were described earlier (Bashan and Levanon, 1990; Bashan and Holguin, 1997). *A. doebereinerae* was found in association with roots of the gramineaeous plant. Strains of the new species are curved rods or S-shaped, 1.0 – 1.5 μm wide and 2.0 – 3.0 μm long. Gram negative, and motile with a single polar flagellum. Nitrogen fixation occurs under microaerobic, nitrogen limited conditions. All these features are very similar to other *Azospirillum* spp. The trait that differentiates this species from others is its ability to use several sugars and some minute genetic details. Optimum growth occurs at 30°C, and at pH values between 6.0 and 7.0, but not at 37°C, as is the case for the other species (Eckert *et al.*, 2001). *A. largomobile* was technically transferred, on the basis of some phylogenetic relationships, from the genus *Conglomomonas* to *Azospirillum* (Dekhil *et al.*, 1997).

*Azospirillum* strains are routinely isolated from agricultural lands and crop plants, including traditional isolations from grasses and cereals (Nath *et al.*, 1997; Weber *et al*. 1999). Apart from direct isolation of potential new strains of *Azospirillum* on various N - free, semi - solid media (Bashan *et al.*, 1993), a new, simple method for isolating *Azospirillum* strains from the
roots and the rhizosphere of rice, based on the capacity of *Azospirillum* to
grow on soil extract medium, was described. Soil extract medium repressed
the most abundant bacterial populations and facilitated isolation of
*Azospirillum* from a population representing <0.001 per cent of the total
microflora (Van *et al.*, 1997).

Beijerinck (1925) first isolated the bacterium from nitrogen poor
sandy soil of the Netherlands and named as *Spirillum lipoferum*. The
bacterium was able to fix atmospheric nitrogen in enrichment culture. In
1963, Becking isolated an “aerobic vibrio or *Spirillum*” from various
Africans soils resembling *Spirillum lipoferum* and confirmed dinitrogen
fixation by the incorporation of $^{15}$N$_2$ gas in liquid medium containing yeast
extract.

The importance of this microorganism was realized when Doberiner and
Day (1976) isolated it from the roots of *Digitaria decumbens*. The
*Spirillum* has been isolated from the roots of numerous wild and cultivated
grasses, cereals, legumes and non-areal crop plants and also from tropical,
sub-tropical and temperate soils, worldwide (Rao and Venkateswarlu, 1982;
Bashan and Levanony, 1990; Fages, 1994).

It is a Gram negative common root and soil inhabitant in the tropics
and so far five species have been described. Both *A. brasilense* and *A.*
*lipoferum* are thoroughly studied among the members of *Azospirilla* and a
number of morphological and physiological characters differentiate between
both species (Tarrand *et al.*, 1978). *A. amazonense*, and an acid tolerant
species was isolated from the grasses and palm trees in Brazil (Magalhaes
*et al.*, 1983) and *A. halopreferens* was isolated from the root surface of
*Leptochloa fusca* in Pakistan (Reinhold *et al.*, 1987).

The occurrence of *Azospirillum* in the rhizosphere varied from 1 to 10
per cent of total rhizosphere population (Okon, 1985). The rhizosphere
contains 100 times more *Azospirillum* than in the non-rhizosphere soils (De Coninck *et al.*, 1988). It has been isolated from different agro climatic zones and in crops all over the world (Michiels *et al.*, 1989). Sumner (1990) reported the occurrence in the rhizosphere of various field grown crops such as rice, wheat, maize, sorghum and pearl millet collected from different locations.

Kabir *et al.* (1995) categorized four species of *Azospirillum* based on the molecular level study using different oligonucleotide probes. Kavitha (2000) isolated *Azospirillum* species from wetland rice and reported that *Azospirillum* accounts for 18 per cent of the total heterotrophic population. The association of *Azospirillum* with cashew and its possible role in improving crop growth and yield was reported by Purushothaman (2002). He isolated over 300 isolates of *Azospirillum* from the root tissues of cashew and characterized them. It appears that *Azospirillum* was a universal bacterium found almost everywhere.

Saleena *et al.* (2002) studied the diversity of *Azospirillum* strains isolated from rice plants grown in saline and non-saline soils of coastal agricultural ecosystem and reported the predominance of *Azospirillum brasilense* and *Azospirillum lipoferum*.

Rao and Charyulu (2003) reported that the rhizosphere of foxtail millet harbored a distinct population of diazotrophic, non-symbiotic bacteria and associative symbiont, *Azospirillum* sp.

Yasmin *et al.* (2004) isolated and characterized plant growth promoting bacteria (PGPB) in four soils and evaluated their potential use as biofertilizers for rice. *Azospirillum* sp. were isolated from the rhizosphere and rhizoplane of certain medicinal herbs *viz.*, *Catharanthus roseus*, *Ocimum sanctum*, *Phyllanthus amarus*, *Coleus forskholii* and *Aloe vera*
(Geetha, 2003; Kalpana, 2005; Karthikeyan et al., 2007; Ezekiel Baudoin et al., 2009).

2.3. Phytohormone production by Azospirillum isolates

Azospirillum spp. are known mainly for their ability to produce plant hormones as well as polyamines and amino acids in culture (Thuler et al., 2003). Among these hormones, indoles, mainly indole acetic acid (IAA) and gibberellins may play a major role.

2.3.1. Indole acetic acid

Confirmatory studies on IAA production by several strains of Azospirillum showed that production depended on the type of culture media and availability of tryptophan as a precursor. A. brasilense Cd produced the highest level of IAA among the strains tested (Radwan et al., 1998). The pH has a significant effect on the amount of IAA produced (Ona et al., 2003). Assessment by chemical methods and with HPLC of possible precursors (indole, anthranilic acid, and tryptophan) for IAA formation in A. brasilense Sp245 revealed a high motive force for tryptophan synthesis from chorismic acid and for IAA synthesis from tryptophan, and this makes it unlikely that anthranilic acid and indole act as the precursors to IAA in a tryptophan-independent pathway. Vitamins may also play a role in the regulation of IAA synthesis in A. brasilense. Very low levels of the B vitamins, especially pyridoxine and nicotinic acid, increased production of IAA in A. brasilense (Zakharova et al., 2000).

Auxin production by Azospirillum sp. is believed to play a major role in plant growth promotion. A. brasilense produced high quantities of extracellular IAA and tryptophol in culture medium supplemented with tryptophan, a precursor of IAA. Addition of filter-sterilized culture supernatants to rice roots grown in hydroponic tanks increased root
elongation, root surface area, root dry matter, and development of lateral roots and root hairs, compared with untreated roots. Higher concentrations of the supernatant strongly inhibited root elongation, lateral root development, and caused nodule-like tumors on the roots (El-Khawas and Adachi, 1999).

Similarly, a cell-free supernatant of *A. brasilense* Cd applied to soybean plants induced the highest number of roots and increased root length (Molla *et al.*, 2001). Inoculation of wheat with *A. brasilense* Sp245 and Sp7 wild strains led to a strong decrease in root length and increase in root hair formation, as is common for such inoculations. The effect on root morphology was further enhanced by adding tryptophan, and this could be mimicked by replacing *Azospirillum* cells with IAA (Dobbelaeere *et al.*, 1999). A mutant of *A. brasilense* with low production of phytohormones, but with high nitrogenase activity did not enhance root growth over uninoculated controls. In contrast, a mutant with increased phytohormone production significantly affected root morphology. In general, increased plant biomass and N$_2$-fixation were recorded in strains having increased production of indole compounds (Kundu *et al.*, 1997).

Induction of para-nodules in rice roots by the auxins 2,4-D, naphthalene acetic acid (NAA), and IAA enhanced polygalacturonase activity in rice roots during formation of the para-nodules and endophytic colonization by *Azospirillum*. While inoculation with *Azospirillum* could augment polygalacturonase activity of rice roots to a small extent without any visible effect on root morphogenesis; auxin application, together with *Azospirillum* inoculation, enhanced polygalacturonase activity of rice roots to a high level, thus, yielding root change into para-nodules that later were colonized by *A. brasilense* (Sekar *et al.*, 1999).
Morphological changes in plant roots following *Azospirillum* inoculation were mimicked by applying a combination of plant growth substances, pointing to the involvement of an auxin produced by *Azospirillum* for root proliferation and consequent plant growth promotion (Bashan and Holguín, 1997). Further evaluation of the contribution of auxin biosynthesis by *A. brasilense* in altering root morphology showed that inoculation of wheat seedlings with an *A. brasilense* Sp245 strain, carrying a mutation in the *ipdc* gene, did not decrease root length nor stimulate root hair formation, in contrast to inoculation with the wild type. These experiments indicate the role of IAA in root proliferation when inoculated with *Azospirillum* (Dobbelaere *et al.*, 1999).

Bloom *et al.*, (2003) have reviewed the signals and molecules that are potentially involved in root development. Among them, nitrogen species as ammonium, nitrate and NO are clearly implicated in root growth and proliferation. In this regard, it has been already demonstrated that NO functions as a signal molecule in the IAA-induced signalling cascade leading to ARD (Pagnussat *et al.*, 2003). More recently, it was also reported that NO plays a central role during lateral root formation (LRF) (Correa-Aragunde *et al.*, 2004), and root hair development (Lombardo *et al.*, 2006).

### 2.3.2. Gibberellins

A beneficial effect of *Azospirillum* spp. on plants has been suggested to be partially caused by the production of gibberellins. Application of gibberellins had effects similar to *Azospirillum* inoculation in increasing root hair density. When *A. lipoferum* USA 5b, a gibberellin-producing strain, was cultured in the presence of glucosyl ester or glucoside of gibberellin A20, both conjugates were hydrolyzed. These *in vitro* results support the hypothesis that growth promotion in plants induced by *Azospirillum* inoculation results from a combination of both gibberellin production and
gibberellin-glucoside/glucosyl ester deconjugation by the bacterium (Piccoli et al., 1997). The effect of water potential or O₂ concentration on growth and gibberellin A₃ (the main gibberellin identified from Azospirillum) production in A. lipoferum showed that gibberellin A₃ produced by each culture was reduced severely at high water potentials or low O₂ concentrations. At the highest water potential concentration, gibberellin A₃ was reduced by only 50 per cent, despite a 90 per cent reduction in cell numbers. This indicates an increase in the amount of gibberellin A₃ produced per cell with increasing water potential (Piccoli et al., 1999). The involvement of gibberellin A₃ produced by Azospirillum spp. In promoting maize growth was also suggested (Lucangeli and Bottini, 1997).

A. brasilense Cd and A. lipoferum USA 5b promoted elongation of root sheaths with 2 single genes in GA deficient dwarf rice mutants, dy and dx, when the inoculated seedlings were supplied with [17, 17-2H₂] gibberellin A20-glucosyl ester. This growth resulted from gibberellin metabolism by the bacteria in the dx mutant, and by both the rice plant and microorganism in the dy mutant. In the dy mutant, inoculation by both bacterial strains reversed dwarfism in seedlings incubated with [17, 17-2H₂] gibberellin A20, forming [17, 17-2H₂] gibberellin A1. It was possible that the bacterial enzyme responsible for these phenomena is 2- oxoglutarate-dependent dioxygenase, similar to those of plants (Cassan et al., 2001).

2.3.3. Nitrogen fixation

Nitrogen fixation was the original proposed mechanism by which Azospirillum affects plant growth, considerable information has already been published (Bashan and Levanony, 1990; Bashan and Holguin, 1997; Kennedy and Islam, 2001). In recent years, only a few studies have focused on the nitrogen cycle within the cell, apart from the genes involved.
The efficiency of N₂-fixation and denitrification in *A. lipoferum* can be regulated by varying the concentration of oxygen, nitrate, and molybdenum. The maximum growth rate in 2 strains was observed under microaerobic conditions (5 per cent O₂), minimal nitrate (2 g/L) and the maximum allowable concentration of molybdenum (0.5 g/L). These conditions also were conducive for the maximum efficiency of denitrification (nitrate reduction to molecular N₂) (Furina *et al.*, 1999). Microaerobic conditions favor N₂-fixation. In *A. brasilense*, cytochrome c oxidase is required under microaerobic conditions when a high respiration rate is needed.

Capability to withstand low temperatures and low oxygen concentrations could depend on the capacity of the bacterium to use nitrate to form nitrite more efficiently. *A. lipoferum* JA03 showed better growth at low temperature (28°C), while *A. brasilense* Sp245 and JA04 grew better at 32°C and 37°C. Additionally, *A. lipoferum* JA03 showed remarkably high nitrite accumulation and more intense growth at low oxygen tension, compared with *A. brasilense* Sp245 and JA04. Perhaps the greatest growth rate at low oxygen tension was partly a result of efficient utilization of nitrate in respiration. These *A. lipoferum* JA03 traits might be adaptive characteristics leading to better acclimation in waterlogged and highly compacted subtropical soils in Brazil (Didonet and Magalhaes, 1997).

Dissolved oxygen was also found to be a limiting factor when ammonium concentrations limit growth of *A. lipoferum* (Tsagou *et al.*, 2003). Out of 40 thermotolerant mutants developed from a mesophilic *A. lipoferum*, only 14 mutants could grow and fix nitrogen at 45°C. These mutants excrete ammonia only as very old cultures. Finally, from an *A. brasilense* that is normally capable of reducing NO₃, a spontaneous mutant was isolated that was defective in both assimilatory and periplasmic
dissimilatory nitrate reductase activity. It was also significantly reduced in its capability to colonize wheat and rice seedling roots. As the rhizosphere is poor in nitrates, functional periplasmic nitrate reductase might be essential for the survival and efficient colonization by *A. brasilense* (Steenhoudt *et al.*, 2001).

Nitrogen fixation was the first mechanism suggested to promote the growth of plants by *Azospirillum*. The majority of evidence collected during the last 3 decades concerning this mechanism has generated controversy. Some greenhouse and field experiments have shown repeatedly that the transfer of nitrogen fixed by *Azospirillum* spp. to the plant is minimal (Bashan and Holguin, 1997; Kennedy *et al.*, 1997). Yet, other studies showed that the bacteria cannot fulfill all of the nitrogen requirements of plants, but nevertheless contribute significant amounts of nitrogen. Furthermore, inoculation commonly and significantly reduced the required doses of nitrogen fertilization in numerous greenhouse and field experiments of many plant species (Bashan and Levanony, 1990; Bashan and Holguin, 1997).

Several confirmatory reports about the contribution of fixed nitrogen by *Azospirillum* to plants, similar in nature to reports of previous years, illustrate the controversy. The 15N isotope dilution technique indicated that there were significant biological N₂-fixation contributions to 2 genotypes of maize that showed similar increases in grain yield when they were inoculated with a mixture of *Azospirillum* spp. Strains or fertilized with the equivalent of 100 kg N/ha. These genotypes showed a large increase in total nitrogen in the plant. This suggests that the yield response resulted from increased nitrogen acquisition, but not from bacterial nitrate reductase; NR-mutants generally caused plant responses similar to those of the parent strains (Garcia de Salamone *et al.*, 1997). The ability of the bacteria to
transfer fixed nitrogen from the atmosphere to wheat plants was tested using 15N₂-enriched atmosphere.

Nitrogenase activity in tumor structures inhabited by bacteria significantly increased, compared with untreated control plants (Christiansen-Weniger, 1997). It is probable that within the para-nodule bacterial nitrogenase activity is less sensitive to increased oxygen tension in the roots. Host plants benefit from enhanced N₂ fixation in their roots with para-nodules because fixed nitrogen is incorporated into host plant material. Host plants probably stimulate nitrogenase activity of *Azospirilla* by providing a carbon source as energy (Christiansen-Weniger, 1998). The effects of inoculating wheat with a highly efficient *A. brasilense* strain under 3 nitrogen regimes revealed that inoculation stimulated plant growth, nitrogen accumulation, and nitrogen and NO₃ − accumulation in the tissues. At maturity, inoculated plants showed higher biomass, grain yield, and nitrogen content than the uninoculated ones, as well as a higher grain protein concentration. It was concluded that *A. brasilense* increased plant growth by stimulating nitrogen uptake by the roots (Saubidet *et al.*, 2002).

On the other hand, using an in vitro model (*A. brasilense* and wheat) within 70 h after inoculation, insignificant amounts of newly fixed N₂ were transferred from an ammonia-excreting strain of *A. brasilense* to the shoot tissue of wheat. By the addition of malate (a preferred carbon source for *Azospirillum*), transfer of nitrogen to the shoots increased 48 - fold, indicating that 20 per cent of shoot nitrogen had been derived from N₂-fixation. Apparently, the inability of the host plant to release sufficient carbon into the rhizosphere is a significant constraint on the development of the *A. brasilense*-wheat association. Perhaps wheat plants with an increased release of photosynthate to the rhizosphere should be a priority for improving the association (Wood *et al.*, 2001).
Nitrogen fixation by aerobic bacteria is a very energy demanding process, requiring efficient oxidative phosphorylation, while O₂ is toxic for the nitrogenase complex. *Azospirilla* and other well known N₂ fixing soil bacteria have evolved a variety of strategies to deal with and overcome this apparent “O₂ paradox”. The question is whether the specific environmental adaptations of *Azospirilla* are sufficient to allow optimal proliferation and N₂ fixation in their natural habitat. Could improving O₂ tolerance of the N₂ fixing process contribute to the development of more efficient strains for inoculation of plants (Marchal and Vanderleyden, 2000).

**2.3.4. Siderophore production by *Azospirillum***

It is believed that siderophore production by *Azospirillum* helps in improved iron nutrition and offers protection against pathogens in plants (Suslow and Sehroth, 1982). Many microorganisms produce siderophores (Kloepfer et al., 1986). Saxena et al. (1986) observed the excretion of phenolate siderophores along with leucine and lysine as well as salicylic acid under iron starved conditions in *A. lipoferum* strain D-2. In *A. brasilense* RG, siderophores of 2, 3, dihydroxy benzoic acid conjugates like spirillobactins were characterized (Bacchawat and Ghosh, 1987).

Shah et al. (1992) characterized the siderophore of *A. lipoferum* and noticed its maximum production after 24 hrs of growth. The antimicrobial activity of siderophores against fungal and bacterial isolates was also observed. They observed that the addition of 1mM FeCl₃ in the iron starved *A. lipoferum* cells resulted in the synthesis of catechol type siderophores. Maximum synthesis was noticed when malate, succinate and gluconate were used as carbon sources but with citrate under aeration inhibited the synthesis of siderophores. *In vitro* addition of siderophores enhanced nitrogenase activity and positive correlation between nitrogen fixation and siderophore production was noticed.
2.4. Enzyme production by *Azospirillum* isolates

Several enzymes in *Azospirillum* have been studied in *vitro* in recent years. *A. brasilense* glutamate synthase, a complex iron-sulfur flavoprotein that catalyzes the reaction of glutamine to L-glutamate, was characterized and transferred to *E. coli* for the overproduction of glutamate synthase holoenzyme. Recombinant *A. brasilense* glutamate synthase was purified to homogeneity after overproduction in *E. coli*, and the purified enzyme was indistinguishable from the original enzyme prepared from *A. brasilense* (Stabile et al., 2000).

The effect of Mg$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ on kinetic properties of GOGAT, a key enzyme of nitrogen metabolism in *A. brasilense* Sp245, revealed that the enzyme level and kinetic behavior of GS depend essentially on the concentration of the ions and their ratios (Antonyuk et al., 2001). Cobalt metabolism in *A. brasilense* Sp245 was studied with emission Mossbauer spectroscopy of Co-57(II)-doped bacterial cells. This technology allowed elements in biological samples to be monitored at their physiological (trace) concentrations. It showed that Co-57(II)-activated glutamine synthetase had 2 different Co (II) forms at its active sites (Kamnev et al., 2002).

Laccase, a *p*-diphenol oxidase that is most typical of plants and fungi, was detected in *A. lipoferum* (Diamantidis et al., 2000). Laccase activity was also detected in a non motile, *in vitro* - grown variant that originated from the motile laccase - negative wild type isolated from rice cultivated under extremely low oxygen concentrations. This stable, atypical variant acquired laccase activity and was capable of producing melanin (Alexandre and Bally, 1999). During the exponential growth phase under fully aerobic conditions, the laccase positive variant lost a respiratory branch that terminated in a cytochrome *c* oxidase, probably from a defect in the biosynthesis of a heme component essential for the oxidase. The laccase-
positive variant was far less sensitive to the inhibitory action of quinone analogs, apparently caused by rearrangements in its respiratory system. It is possible that the loss of the branch containing cytochrome c oxidase in the variant is an adaptation to the presence of intracellular, oxidized quinines produced by laccase (Alexandre et al., 1999). The ecological significance of these Azospirilla is yet to be discovered.

Eukaryote and prokaryote microorganisms contain β-glucosidases that catalyze the hydrolysis of cellobiose and chemically related β-glucosides. A. irakense KBC1 grows on pectin and β-glucosides, such as cellobiose, arbutin, and salicin. Glucose released after biological cleavage of aryl-β-glucosides is a suitable carbon source for many bacteria, and could contribute to the competitiveness and survival of bacteria in the rhizosphere (Faure et al., 1999).

A. irakense isolated from surface sterilized, field grown rice roots suggested that the bacterium can penetrate plant roots and use cell wall degrading enzymes (Khammas et al., 1989). Two new pectate lyase enzymes were isolated and characterized from A. irakense (Bekri et al., 1999; De Armas et al., 2002), the sole source of these enzymes in the genus Azospirillum. The A. irakense pelA gene that encodes a pectate lyase was isolated by heterologous expression in E. coli (Bekri et al., 1999). Pectin stimulated pelA transcription significantly. An A. irakense pelA-Tn5 mutant still displayed pectate lyase activity, suggesting multiple pectate lyase genes in A. irakense (Bekri et al., 1999).

2.5. Mechanism of growth promotion by Azospirillum

Several mechanisms, other than Biological Nitrogen Fixation (BNF) mentioned above, have been postulated to explain how Azospirillum enhances growth and development of plants, such as phytohormone production and nitrate reduction (Steenhoudt and Van der Leyden, 2000).
Nevertheless, to date no unique mechanism had been established to explain the growth promotion capability of these bacteria. Instead, the most accepted hypothesis postulates that a sum of events accounts for the general plant growth promotion effect (Bashan and Holguin, 1997).

*Azospirillum* spp. is not considered to be a classic biocontrol agent of soil-borne plant pathogens. However, there have been reports on moderate capabilities of *A. brasilense* in biocontrolling crown gall-producing *Agrobacterium* (Bakanchikova *et al.*, 1993); bacterial leaf blight of mulberry (Sudhakar *et al.*, 2000); and bacterial leaf and/or vascular tomato diseases (Bashan and de-Bashan, 2002). In addition, *Azospirillum brasilense* can restrict the proliferation of other non-pathogenic rhizosphere bacteria (Holguin and Bashan, 1996). These *Azospirillum*’s antibacterial activities could be related to its already known ability to produce bacteriocins (Oliveira and Drozdowicz, 1987) and siderophores (Tapia- Hernández *et al.*, 1990; Shah *et al.*, 1992).

In addition, it was recently reported that *A. brasilense* can synthesize phenylacetic acid (PAA), an auxin-like molecule with antimicrobial activity (Somers *et al.*, 2005). Phenylacetic acid was detected by concentration of culture supernatant only in the presence of 0.5 mM phenylalanine added to the media as a precursor molecule. It was also detected at the onset of secondary metabolism. However, since the authors worked only with concentrated supernatant extracts, it is yet unknown if the production of phenylacetic acid by *Azospirillum brasilense* under *in vitro* is sufficient to be considered of ecological importance (Somers *et al.*, 2005).

As a primary target, the root is the organ that shows the first stimulating bacterial effects. This was particularly remarkable in plants inoculated with *Azospirillum* spp. (Okon, 1985). Upon inoculation the root displayed a significant increase in the number and the length of root hairs,
the rate of appearance and number of lateral roots, the diameter and length of lateral and adventitious roots and the root surface area (Creus et al., 2005).

The increased root development leads to an increased root surface that could improve plant nutrition and thus would be a key factor for plant growth promotion by PGPR in general. In this sense, developmental changes promoted in roots must be triggered prior to the changes in uptake of nutrients. This widely accepted hypothesis also states that nutrient uptake would be increased over time together with increased root surface. In this view, nutritional improvement by PGPR would be an indirect consequence of their effect on root development (Mantelin and Touraine, 2004).

Bertrand et al. (2000) showed that an Achromobacter sp. Enhanced NO$_3^-$ uptake rate per unit of root area in Brassica napus roots, and Saubidet et al. (2002) reported that the inoculation with Azospirillum brasilense increased the nitrogen content of wheat plants. Bashan et al. (1992) showed that soybean and cowpea inoculated with Azospirillum brasilense enhance H$^+$ extrusion from their roots compared to the normal extrusion occurred in non-inoculated plants. After nine hours of transferring cowpea plants growing in hydroponic solutions to a new one the pH of the media decreased from 6 units of pH to the range of 4.13 ± 0.19 units, providing more evidence about a direct effect on root cell membranes. In addition to its physiological activities on root membranes, there is evidence that fatty acid composition of main phospholipids in roots is affected by inoculation by Azospirillum brasilense (Pereyra et al., 2006).

It has been assumed that all the Azospirillum effects on plants are dependent on the plant species and cultivar inoculated and on the inoculum concentration used (van de Broek et al., 2000). Regarding the last mentioned factor, inoculation of many different plant species with Azospirillum in a
range between 106 to 108 cells per seedling provoked root elongation (Creus et al., 1996). However, higher concentrations of bacteria Azospirillum brasilense always results in restricted root growth (Pereyra et al., 2007). Thus, there exists an optimum bacterial concentration for triggering root elongation. The production of plant growth substances by Azospirillum has often been proposed as one of the key factors responsible for the observed plant growth promotion, as plant growth substances could be detected in the supernatant of Azospirillum cultures.

Tien et al. (1979) showed that Azospirillum is able to produce auxins when exposed to tryptophan. In fact, a variety of auxins like indole-3-acetic acid (IAA), indole-3-pyruvic acid, indole-3-butyric acid and indole lactic acid (Martinez-Morales et al., 2003); cytokinins (Cacciari et al., 1989) and gibberellins (Bottini et al., 1989) were detected, with auxin production being quantitatively most important. Studies on IAA production showed that it relay on the type of culture media and availability of tryptophan as a precursor. Among the strains tested A. brasilense Cd produced the highest level of IAA (El-Khawas and Adachi, 1999).

Combinations of different amounts of indole acetic acid, gibberellin, and kinetin all of them in ranges of 0.001 to 0.05 μg ml⁻¹ produced changes in root morphology of pearl millet similar to those produced by inoculation with Azospirillum brasilense (Jain and Patriquin, 1984). In addition, the effect of Azospirillum inoculation on root elongation of wheat plants (Dobbelare et al., 1999) and on branching of wheat root hairs (Jain and Patriquin, 1984) was also mimicked by the application of IAA to roots. Application of increasing concentrations of IAA to wheat seeds strongly decreased wheat root length, comparable to the effect seen upon inoculation with increasing concentrations of bacteria (Dobbelare et al., 1999). Treatment with IAA can be compared to inoculation with 10⁸ bacteria per
ml, showing increased number and length of root hairs and a decreased root elongation zone as compared to control roots (Dobbelaere et al., 1999).

The production of these regulators, in turn, could be influenced by compounds released by plant roots (Omay et al., 1993). As well as sugar released by roots is a C-source in feeding bacteria, it could also stimulate auxin synthesis in Pseudomonas spp. (Leinhos, 1994). In this regard, the same root exudates that in vivo induce IAA production by Pseudomonas fluorescens could be also stimulating in the rhizosphere the hormonal release near roots (Benizri et al., 1998). Anyway, the possibility that Azospirillum could not only produce IAA but also enhance the endogenous IAA produced by the plant should not be excluded. Most studies on mechanisms for plant growth promotion by PGPR have focused on bacterial traits without examining the host plant’s physiological responses (Bloemberg and Lugtenberg, 2001). Moreover, the role of chemical signals in mediating belowground interactions is only beginning to be understood (Bais et al., 2006).

Bloom et al. (2003) have reviewed the signals and molecules that are potentially involved in root development. Among them, nitrogen species as ammonium, nitrate and NO are clearly implicated in root growth and proliferation. In this regard, it has been already demonstrated that NO functions as a signal molecule in the IAA-induced signalling cascade leading to ARD (Pagnussat et al., 2003). It was also reported that NO plays a central role during lateral root formation (LRF) (Correa-Aragunde et al., 2004), and root hair development (Lombardo et al., 2006).

It has been largely known that Azospirillum can produce NO at low O₂ pressure by denitrification (Hartmann and Zimmer, 1994). The remarkable analogies found between the experimental data concerning Azospirillum stimulation of plant root development and the capability of NO
to act as a non-traditional plant growth regulator (Beligni and Lamattina, 2001) promoting ARD, LRF and root hair initiation and elongation, led us to explore whether the *Azospirillum* ability to promote root growth and change its architecture relies on NO.

Creus *et al.* (2005) reported the NO production by this microorganism growing under aerobic conditions. A concentration of 6.4 n moles of NO per gram of *Azospirillum brasilense* was quantified when the bacterium reached the end of growing log phase. In addition, *Azospirillum* inoculated tomato roots incubated with a NO-specific fluorescent probe displayed higher fluorescence intensity compared to non-inoculated roots. Fluorescence was mainly located at the vascular tissues and sub-epidermal cells of roots (Creus *et al.*, 2005). Moreover, the *Azospirillum*-mediated induction of LRF appears to be NO-dependent since treatment of inoculated seedlings with the NO scavenger- (4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl- 3-oxide completely blocked this effect (Creus *et al.*, 2005).

### 2.6. *Azospirillum* and salt stress

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable in India. It contributes significantly to the nutrition of the people of this country as a source of vitamins and minerals. The production of tomato is constrained in the coastal area of India due to lack of knowledge of improved technology and upward or lateral movement of saline groundwater during the dry season *viz.*, November - May (Pal *et al.*, 1994). Salinity is an important determinant for soil capability and it is seen as a “modifier” which put restrictions on possible crop choices (Wilde, 2000).

Escaping or minimizing soil salinity is very important for the tomato cultivation in the saline soil. Any practices that reduce evaporation from soil surface and or encourage downward flux of soil water will help to control root zone salinity. Mulch is one the effective management means to reduce
dry season salinity and conserve moisture in the root zone. This practice also encourages deeper and denser rooting. Reduction of soil salinity and 36.32 per cent more tomato fruit yield was recorded by mulch over control (Anonymous, 2000). Mulches have been found to decrease soil moisture losses by reducing soil temperature and evaporation, promoting favorable soil biotic activities, reducing hard soil setting and contributing plant nutrients (Pal et al., 1994).

Numerous cultivated soils worldwide are becoming more saline mainly from the use of marginal irrigation water, from excess fertilization, and various desertification processes. Inoculation with Azospirillum sp. under saline stress conditions is therefore commonplace. Prior findings showed that common agricultural Azospirillum strains tolerated high salinity (≤2%). Salt resistance among species increased from A. amazonense (lowest) to A. halopraeferens (highest), the latter tolerating over 3% NaCl (seawater salinity). The common cellular mechanism of osmotic stress adaptation is intracellular accumulation of organic solutes. In sorghum, inoculation with A. brasilense diminished the adverse effects caused by osmotic stress (Bashan and Holguín, 1997).

More recent and detailed confirmatory in vitro studies demonstrated that A. brasilense Cd can tolerate up to 200 m mol/L NaCl in the medium without appreciable decline in growth. Higher concentrations of salt caused inhibition of bacterial growth. At 300 m mol/L NaCl, growth decreased 66% after 24 h. After 48 h at this concentration, bacteria reached maximum optical density, comparable to the optical density of control cultures after 12 h. A. brasilense responded to saline stress by elevating intracellular concentrations of glutamate at 24 h and K+ at 48 h.

Although, several cellular functions are affected by saline stress, it seems that A. brasilense Cd has remarkable tolerance for saline conditions
(Rivarola et al., 1998). As already known, *Azospirillum* spp. can accumulate compatible solutes, such as glycine betaine, glutamate, proline and trehalose, to allow adaptation to fluctuations in soil salinity/osmolarity. Addition of these osmo-protectants to bacterial cultures under saline stress usually increased cellular growth and nitrogenase activity (Choi and Gal, 1998; Tripathi and Mishra, 1998). As inhibition of *A. lipoferum* growth by NaCl was relieved by exogenous glycine betaine, this suggested that *A. lipoferum* has a salinity-induced glycine betaine transport system (Tripathi and Mishra, 1998). Additionally, proline seems to play a major role in osmoadaptation.

With increases in osmotic stress, the dominant osmolyte in *A. brasilense* shifted from glutamate to proline. Accumulation of proline occurs by uptake and synthesis. At higher osmolarity, *A. brasilense* Sp7 accumulated a high intracellular concentration of glycine betaine that was taken up via the glycine betaine transport system, probably similar to that of *A. lipoferum*. Except for *A. halopraeferens*, all other species of *Azospirillum* lacked the ability to convert choline to glycine betaine. Mobilization of the *betABT* genes of *E. coli* in *A. brasilense* enabled it to use choline for osmo-protection (Tripathi et al., 1998).

Saline stress alters *A. brasilense* Cd maize and wheat interactions, normal colonization, and N₂-fixation. Saline stress altered the early stages of plant development, which led to inadequate colonization and expression of *A. brasilense* *nif* gene promoters. Attachment of *A. brasilense* Cd to maize and wheat roots was altered when the bacteria were grown under saline stress. Alteration in the adsorption phase of attachment appeared to be related to the disappearance of a 100 kD external membrane protein in the bacterium. Gene expression in *A. brasilense* was influenced by the presence of plant root exudates; wheat root exudates induced the reappearance of the 100 kD protein (Fischer et al., 1999). Unstressed bacteria grown under
standard conditions were distributed over the entire root system of wheat, except the elongation zone. Bacteria subjected to saline stress were mainly found at the root tips and the lateral roots. Interestingly, salt treatment reduced surface colonization, but not colonization inside the root (Fischer et al., 2000).

*Azospirillium* inoculation at NaCl concentrations up to ~1.2 MPa significantly increased chlorophyll, K, Ca, soluble saccharides, and protein contents as compared with control plants growing without NaCl (Hamdia and El-Komy, 1997), similar to alleviating water stress on wheat plants grown under drought conditions (El-Komy et al., 2003). Inoculating *A. brasilense* on wheat seedlings exposed to severe salt (NaCl) or osmotic (polyethylene glycol) stresses significantly reversed part of the negative effects; both stresses reduced relative elongation rate of shoots. Fresh weight, fresh wt/dry wt, water content, and relative water content were higher in shoots from inoculated plants than in stressed controls (Creus et al., 1997). Turgor pressure at low water potential was higher in inoculated seedlings in 2 wheat cultivars under osmotic stress. This could result from better water uptake as a response to inoculation that, in turn, is reflected by faster shoot growth in inoculated seedlings exposed to these stresses. These results are similar to what was found in water-stressed wheat inoculated with *Azospirillum*. They showed better water status and effects on cell wall elasticity and (or) apoplastic water (Creus et al., 1998).

Soil salinity plays a major role in diversity of indigenous *Azospirilla* associated with rice along the coast of India. All the strains detected were *A. brasilense* or *A. lipoferum*. Decreasing diversity of *Azospirillum*, probably from higher salinity, is apparently more important than the clay fraction of the soil, a factor demonstrated as having a major role in several studies. If true, this association between soil salinity and range of *Azospirillum* should
be considered when developing inoculants, especially for coastal salt affected agricultural areas (Saleena et al., 2002).

The effect of salt on *Azospirillum*-plant interactions is a major factor to be considered in applied studies. In spite of its importance, meagre molecular studies have been conducted on the bacterium per se, and almost none as to interactions with plants. The most fundamental omission in current knowledge is uncertainty on whether improved salt tolerance of the bacterium is needed to enhance the bacterium’s effect on plants or if existing salt tolerance is adequate to ensure positive growth promotion by inoculation.

2.7. *Azospirillum* – plant interactions

*Azospirillum* spp. is included into the alpha subclass of Proteobacteria belonging to the IV rRNA superfamily (Xia et al., 1994). This group of free-living rhizobacteria encompasses ten species, each one classified according to its particular biochemical and molecular characteristics: *A. lipoferum* and *A. brasilense* (Tarrand et al., 1978); *A. amazonense* (Magalhaes et al., 1983); *A. halopraeferens* (Reinhold et al., 1987); *A. irakense* (Khammas et al., 1989); *A. largimobile* (Dekhil et al., 1997); *A. doebereinerae* (Eckert et al., 2001); *A. oryzae* (Xie and Yokota, 2005); *A. melinis* (Peng et al., 2006) and recently *A. canadensis* (Mehnaz et al., 2007). Although, *Azospirillum* was first isolated from cereals and most of the initial inoculation has been done on the main cereal crops, there are more non-cereal species successfully inoculated with *Azospirillum* than cereals. *Azospirillum* strains have no preferences for crop plants or weeds, or for annual or perennial plants, and can be successfully applied to plants that have no previous history of *Azospirillum* in their roots. It appears that *Azospirillum* is a general root colonizer and not a plant specific bacterium (Bashan and Holguin, 1997; Bashan et al., 2004).
*Azospirillum* is not the only microorganism capable of colonizing vegetables and inducing beneficial effects on them, but it congregates several characteristics present in different microorganisms, that make it a valuable PGPR. Indeed, both a higher growth and yield observed in *Azospirillum*-inoculated sub-tropical grasses (*Z. mays, O. sativa, Saccharum officinarum, Sorghum bicolor* and forages such as *Digiaria* spp.) were primarily attributed to the biological N\textsubscript{2} fixation (BNF) exerted by the bacteria (Dobereiner and Day, 1976).

The process is performed by a nitrogenase complex, and occurs when the availability of nitrogen compounds and oxygen tension are low (Steenhoudt and Vanderleyden, 2000). Even though, this characteristic could be extremely valuable in agriculture, later field studies including those in which isotopic dilution techniques were used, failed to demonstrate a significant BNF in *Azospirillum*-inoculated crops (Van de Broek et al., 2000). Even at the lab level, the growth promotion effect induced by the inoculation of axenic seedlings could not be ascribed to BNF (Bashan et al., 1989).

Further studies pinpointed the positive bacterial effects on plants on morphological and physiological changes in the inoculated roots that would lead to an enhancement of water and mineral uptake (Okon and Kapulnik, 1986). Other physiological changes observed in the inoculated plant subjected to abiotic stresses were reported. *Azospirillum* - inoculated wheat seedlings subjected to osmotic stress developed significant higher coleoptiles, with higher fresh weight and better water status than non-inoculated seedlings (Alvarez et al., 1996; Creus et al., 2005).

Taking into account that a plant exposed to salt stress also suffer water deficit, it was proved that inoculating with $10^8$ cells of *A. brasilense* on root seedlings and thereafter exposed to mild and severe salt stress
significantly reversed part of the negative effects. *Azospirillum* - inoculated wheat seedlings were able to survive when exposed to up to 320 mM NaCl for three days (Creus et al., 2004). Uniform wheat seedlings were inoculated with *A. brasilense* Sp245, performed by dipping roots in a $10^8$ bacterial cells ml$^{-1}$ suspension for 3 h. After that, the inoculum was replaced either by distilled water, 160 mM NaCl, 320 mM NaCl, 20 per cent PEG 6000, or 30 per cent PEG 6000, and seedlings were grown at 20°C in a growth chamber in the dark up to 3 days.

Fresh weight, fresh weight/dry weight, water content, and relative water content were higher in shoots from inoculated plants than in stressed controls (Creus et al., 2005). These changes could be explaining in part a better performance of crops. Indeed, field experiments carried out with *Azospirilla*-inoculated *S. bicolor, Z. mays* and *T. aestivum* have shown significantly increased yields accompanied by better water and mineral uptake, less canopy temperature and improvement in growth and yield (Casanovas et al., 2003; Creus et al., 2004).

An early review on the benefits a plant could obtain following *Azospirillum* inoculation stressed the importance of improving plant-water status for plants. In this sense inoculation technology with *Azospirillum* could be extended to arid soils in order to protect crops against drought. As the main effect of *Azospirillum* is to promote a more developed radical system, plant adaptation to water stress could be enhanced in inoculated crops. In this regard, experiments concerning the response mechanisms of plants to water stress demonstrated that significantly higher water content, relative water content, water potential, apoplastic water fraction, and lower cell wall modulous of elasticity values were obtained in *Azospirillum*-inoculated plants suffering drought. Yield loss due to stress diminished in *Azospirillum*-inoculated wheat and grains had significantly 38.4; 22.2 and
125 per cent higher Mg, K and Ca respectively, than non-inoculated plants (Creus et al., 2004).

Anyway, it is agreed that the beneficial *Azospirillum* effects on plants relies upon good root colonization. If a positive effect of inoculation with *Azospirillum* sp. is expected, significant root colonization should happen first. Root colonization is important as the first step not only in infection by soil-borne pathogens but also in beneficial associations with microorganisms. The first event in the colonization process is the attachment of bacteria to roots. In the *Azospirillum* - root interaction, this is a two step process comprised of adsorption, mediated by bacterial proteins, and anchoring involving bacterial polysaccharides (Michiels et al., 1991). To attach and colonize plant root surfaces *Azospirillum* spp. must first rely in a process that depends on active motility and chemotaxis toward root exudates.

The distribution of *Azospirillum* in the root was studied with different techniques. Using the gfp-protein to tag bacteria Liu et al. (2003) confirmed previous findings about colonizing patterns. The bacteria is established mainly on the root surface but some strains of *A. lipoferum* and *A. brasilense* but not others are capable of colonizing the root interior in the apoplast and intercellular spaces. This ability could mean a lower vulnerability to harsh conditions imposed by the soil and/or the environment, which in turn could imply a more efficient promotion of plant growth (Sturz and Nowak, 2000). In this regard, rhizobacteria established inside roots in intimate association with plants are considered endophytes. These microorganisms live outside the symplast and do not produce nodules, but can produce signal compounds that stimulate plant growth, enhance plant disease resistance, or improve mobilization of soil nutrients.
2.8. Effect of *Azospirillum* isolates on agricultural crops

The nitrogen fixing rhizobacterium *Azospirillum* lives in close association with plant roots, where it exerts beneficial effects on plant growth and yield of many crops of agronomic importance. The effect of *Azospirillum* inoculation on the total yield increases of field grown plants generally ranged from 10 to 30 per cent (Rao and Venkateswarulu., 1982; Watanabe and Lin, 1984). Bashan *et al.* (1989) reported that 70 to 75 per cent of pot experiment in cotton and several vegetables resulted in yield increase.

Wani (1990) evaluated the worldwide success of *Azospirillum* inoculation and concluded that positive effects on yield were obtained in approximately 65 per cent of field experiments. In several countries, the inoculation of *Azospirillum* in the field studies have shown increased yields of cereals.

*Azospirillum* increased the grain yield and dry matter marginally by 2 to 10 per cent over the uninoculated control (Okon and Labandera-Gonzalez, 1994). Fulchieri and Frioni (1994) observed that maize (*Zea mays*) inoculated with *Azospirillum* has enhanced dry weight of seeds by 59 per cent and also the yield, which was similar to 60 kg urea ha⁻¹.

Significant yield increase was reported in tomato due to the inoculation of *Azospirillum* (Bashan and Holguin, 1995). Bashan and Dubrovsky (1996) recorded higher dry matter production in grasses as influenced by the application of *Azospirillum*. Inoculation of *Azospirillum* with 75 per cent recommended dose of N was superior to uninoculated control in increasing the yield of cumbu variety UCC-5 (Nirmala and Sundaram, 1996). Salomone and Dobereiner (1996) also found increased yield in maize when inoculated with *Azospirillum*. 
*Azospirillum* increased the plant height, grain and fodder yield and also 25 per cent saving in the N fertilizer was observed in Rabi sorghum (Alagawadi and Gaur, 1998). Gadagi *et al.* (2003) reported that inoculation of nitrogen fixing *Azospirillum* increased plant growth parameters and flower yield in Chrysanthemum, Marigold and China aster. Tamizhvendan and Subramanian (2000) reported that application of *Azospirillum* during first and second earthing up resulted in higher growth and kapas yield of rice fallow cotton (ADT-1). Ponnuwashamy *et al.* (2002) recorded increased grain yield of 4830 Kg ha\(^{-1}\) in rainfed sorghum by the application of recommended NPK, FYM and *Azospirillum*.

Soil inoculation using *Azospirillum* improved seed germination, biometric characters and biochemical attributes such as chlorophylls, soluble carbohydrates, reducing sugars, total free amino acids, buffer soluble proteins and phenolics in silk cotton (Vijayakumari and Janardhanan, 2003).

Rueda-Puente *et al.* (2004) reported that the pickle weed (*Salicornia bigelovii*) exhibited increases in growth parameters, biochemical characteristics, including total protein, ash and lipid content and yield parameters, when inoculated with *A. halopraeferens*. Seed inoculation with *Azospirillum* and high levels of nitrogen resulted significantly higher yields and increase in total N and total lipids content of the maize seeds in comparison to the control.

The effect of *Azospirillum* on the total yield increase of field grown plants generally ranges from 10 to 30 per cent (Watanabe and Lin, 1984). Several reports suggest that PGPR stimulate the plant growth by facilitating the uptake of nutrients like N, P and K and micronutrients by the plant. *Azospirillum* sp. enhanced nutrient uptake from soil solution at faster rates and accumulated as dry matter at higher rates (Sarig *et al.*, 1984; Yahalam *et al.*, 1984).
Pectinolytic activity of *Azospirillum* cells contributed to an increased mineral uptake, which might be due to the hydrolysis of the middle lamellae without causing host cell collapse and accelerated water and nutrient uptake by the roots (Kapulink *et al.*, 1985). Bekri *et al.* (1999) also showed substantial pectinase activity in *A. irakense* and explained their role of the enzyme in increasing the *Azospirillum* root colonization.

Inoculation with efficient strains of *Azospirillum* enhanced the yield of CSH 5 and CO 24 sorghum while K-tall and USH1 showed negligible response (Purusothaman and Oblisami, 1986). During the past twenty years, numerous reports of inoculant effect of *Azospirillum* on all kinds of crop plants have been published (Sumner, 1990 and Fages, 1994). A few reports indicate extremely higher values of 50-270 per cent increase in yield over uninoculated controls (Bashan and Levanony, 1990). Moderate yield increase of 20 per cent attributed to inoculation with *Azospirillum* was considered commercially valuable to modern agriculture, if obtained consistently.

Okon (1985) and Wani (1990) evaluated the world wide success of *Azospirillum* inoculant and concluded that positive effects on yield were obtained in approximately 65 per cent of field experiments and in about 70 to 75 per cent of pot culture experiments in several vegetables (Bashan *et al.*, 1989).

Plant height, number of primary and secondary branches and number of leaves increased in Coleus parviflorus by application of 60Kg N ha\(^{-1}\) along with *Azospirillum* 2 Kg ha\(^{-1}\) at the time of planting. However, application of 100 Kg N ha\(^{-1}\) along with 2 Kg ha\(^{-1}\) of *Azospirillum* increased the foliar N, P, Ca and Mg (Nageswari, 1991).

The improvement in plant height, number of leaves, leaf area index, shoot and dry matter was better in *C. parviflorus* inoculated with
*Azospirillum* at 4 Kg ha$^{-1}$ at planting. Tuber yield and harvest index were also the highest (Nageswari and Pappiah, 1993). The seed treatment and seedling treatment with *Azospirillum* increased the growth parameter, ash content and total alkaloids in Ashwagandha (Ramesh Babu, 1996).

Kalyani *et al.* (1996) reported that soil inoculation of *Azospirillum* coupled with less nitrogen of 80 Kg ha$^{-1}$ had beneficial effect in improving the growth and yield of cauliflower cv. Jawahar Moti, besides saving recommended nitrogen upto 50 per cent.

Inoculation of different wheat cultivars with the most efficient strain for N$_2$- fixation resulted in increased growth and nitrogen to the 5 cultivars tested but the effect varied among the cultivars. These results suggest that a potential exists for *A. brasilense* to supply considerable nitrogen to wheat plants, probably dependent on bacteria-cultivar interaction (Saubidet *et al.*, 1998).

In culture medium, wild strain of *A. brasilense* was able to produce indole-3 butyric acid. The compound obtained from culture filtrate was able to increase growth of maize seedlings when sprayed on the crop under *in vitro* conditions (Martinez-Morales *et al.*, 2003).

Early germination, maximum shoot length, increased number of leaves and leaf area in radish was observed in the treatment which received *Azospirillum* inoculant with recommended close of 75 per cent N and P and 100 per cent K (Kamalakannan and Manivannan, 2003).

The inoculation of *Azospirillum* along with 37.5 Kg N and 25 Kg P ha$^{-1}$ increased the plant height, number of leaves, number of laterals and root diameter fresh and dry weight of root, number of berries and seed yield per hectare in Ashwagandha (Navamani and Bharathi, 2002). The maximum
increase in growth parameters and fruit and seed yield of Ashwagandha treated with *Azospirillum* alone was recorded (Gopal, 2004).

Hoshang Naserirad *et al.* (2011) investigated the effects of bio-fertilizer on yield and its components of maize cultivars. Treatments were cultivar factor as main plots and bio-fertilizer factor (non-inoculation, inoculation with *Azotobacter, Azospirillum* and double inoculation of *Azotobacter* and *Azospirillum*) as subplots. Cultivar of SC704 had the highest plant height (201.1 cm), number of grains per row (42.8 grains), grain yield (10850 kg ha\(^{-1}\)) and biological yield (22040 kg ha\(^{-1}\)) compared with other cultivars.

Double-inoculation of *Azotobacter* and *Azospirillum* had the highest plant height (212.4 cm), stem diameter (2.5 cm), number of rows per ear (14.5 row), number of grains per row (44.2 grain), 1000-grain weight (315.4 g), grain yield (10190 kg ha\(^{-1}\)), biological yield (21320 kg ha\(^{-1}\)) and protein content (10.7 per cent) when compared with other treatments. The interaction effect of cultivar × plant growth promoting rhizobacteria (PGPR) on grain yield, biological yield and protein content was significant (p<0.01). The highest and lowest grain yield obtained from SC704 with double inoculation of *Azotobacter* and *Azospirillum* (12320 kg ha\(^{-1}\)) and SC 604 with non inoculation treatment (12320 kg ha\(^{-1}\)), respectively.

Melvin Joe *et al.* (2012) compared the efficiency of *Azospirillum brasilense* flocculated cells with standard grown cells under *in vitro* conditions and in association with maize (*Zea mays* L.) under field conditions. The adhesion efficiency of *A. brasilense* flocculated cells was 54 per cent higher to hydrophobic polystyrene and 101 per cent higher to maize roots when compared to standard grown *A. brasilense* cells. Furthermore, flocculated cells had better spermoplane survivability (48 per cent) and spermosphere colonization (73 per cent) along with a concomitant
enhancement on the germination percentage (11 per cent) and vigour index (23 per cent) of maize. Field studies with A. brasilense flocculated cells conducted under normal irrigated conditions and by withholding irrigation at 25, 50, and 75 per cent available water-holding capacity showed a significant increase in plant height (19 per cent), plant dry weight (16 per cent), grain yield (31 per cent), stover yield (17 per cent) and nitrogen uptake (18 per cent) compared with standard grown cell treatment.

Noshin Ilyas et al. (2012) isolated and characterized Azospirillum strains from maize (Zea mays L.) grown under well watered and water stressed conditions and to evaluate the ability of bacteria to produce plant growth promoting hormones like IAA, Gibberellic Acid, trans-Zeatin riboside and abscisic acid. A total of eight strains of Azospirillum were isolated from rhizosphere and roots of maize plants grown in pots and it was observed that survival efficiency of Azospirillum from well watered plants was higher as compared to that of Azospirillum strains isolated from roots and rhizosphere samples of water stressed plants (having 8-12 per cent soil moisture). Inoculation of wheat with isolates from water-stressed plants induced tolerance to water stress in inoculated plants. Isolates from water-stressed conditions produced low concentration of indole acetic acid, gibberellic acid, and trans zeatin riboside but higher concentration of abscisic acid. The isolated bacterial strains have technological implications for inoculants formulation and improved growth of cereal crops.

2.9. AM fungi

The term mycorrhiza, which literally means “fungus root”, was first applied to fungus tree associations described in 1885 by the German forest pathologist A. B. Frank. Since then, it was recorded that vast majority of terrestrial plants, form symbiotic associations with these fungi. This symbiosis was characterized by bi-directional movement of nutrients where
carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant, root and soil (Gerdemann, 2008).

Endomycorrhizal fungi produce “arbuscules” and “vesicles”, where arbuscules are believed to function in the bidirectional transfer of nutrients between the symbiotic partners and vesicles are the storage organs produced at the tip of the hyphae (Scannierini and Bonfante Faslo, 2003). Of the several kinds of mycorrhizae, the vesicular arbuscular type is by far the most common (Gerdemann, 2008). In the past, VAM fungi are renamed as arbuscular mycorrhizal fungi (Morton, 1998).

Arbuscular mycorrhiza (AM) fungi shown in are geographically ubiquitous. They are commonly found in association with agricultural crops, shrubs, tropical tree species and some temperate trees. Their nutritional requirements are not specific. AM associations are formed by non septate Zygomycetes and Phycomycetes fungi. Some examples are Glomus, Gigaspora, Acaulospora, Entrophospora and Scutellospora of which Glomus is the most common fungus (Remy et al., 1994).

Arbuscular mycorrhizal (AM) fungi occur in most vegetation types and constitute an important component of the tropical soil microflora (Cardoso and Kuyper, 2006). They have been shown to increase growth and yield of plants. They are also found to have positive effects on plants, such as increased resistance to pathogens (Hampp et al., 1999), drought (Shi et al., 2002) or heavy metal stress (Blaudez et al., 2000). They have been identified to play roles in both nutrient mobilization and nutrient cycling. Their distribution and diversity in tropical ecosystems elsewhere appears to be receiving increased attention (Lovelock and Ewel, 2005).

There were many reports on the association of AM fungi in several cultivated crops (Lakshman and Raghavendra, 1995). However, the number
of studies on the influence of AM fungal status on fodder crop plants is limited in India particularly in Tamil Nadu. AM fungi are known to play an important role in the growth and development of fodder crop plants, as they help increase the uptake of diffusion limited nutrients (Palipane and Bandara, 1985). Inoculation of AM fungi improves the physiological conditions of fodder crops.

AM fungi occur as communities in soil and in roots, they are likely to collectively contribute to nutrient uptake, such as P (Jakobsen et al., 2001). Joner and Leyval (2001) suggested that the use of AM fungal consortia adapted to metal-enriched soil rather than single AM fungal species should be considered in the future investigation on the effect of AM fungi. Further, as AM fungi coexist and interact with Plant Growth Promoting Rhizobacteria (PGPR) in soils, changes in microbial community structure may also affect the function of AM fungi, which necessitates experiments with unsterilized soils.

Arbuscular mycorrhiza forms a beneficial interaction with a wide range of plants, including horticultural and forage field crops. The fungi promote efficient nutrient absorption and increase plant growth and yield. Arbuscular mycorrhiza interactions with the soil play an important role in controlling soil fertility, soil erosion and plant water stress. Overall, using Arbuscular mycorrhiza fungi in crop production should reduce fertilizer amounts applied to farmland. The importance of Arbuscular mycorrhiza fungi to sustainable agriculture and the ecosystem has led to its commercial development. However, for the past thirty years, progress on Arbuscular mycorrhiza cultivation has not been very successful, partly because the research is very difficult and time consuming. ARS researchers have found optimal conditions for propagating the dual culture of root and fungus. Pure
and clean Arbuscular mycorrhiza fungi can be grown on gel plates in a completely reproducible manner (Gerdemann, 2008).

The Arbuscular mycorrhiza fungi are the best known for their ability to improve plant growth in low phosphate soils by exploiting large areas of soil and actively transporting the phosphate back to the plants. Other benefits to the plants supplied by the Arbuscular mycorrhiza colonization include increased absorption of nitrogen, potassium, magnesium, copper, zinc, boron, sulphur, molybdenum and other elements that are transported back to the plant. Finally, it helps in retaining moisture around root zone of plants (Srihari, 1997).

Most plants have more than roots, they have Arbuscular mycorrhiza. They are fungi that live in a harmonious relationship with plant roots. This is a symbiosis in which the fungi provide the plant with extra nutrients from the soil, especially phosphorus and zinc, in exchange for sugars (exudates) provided by the plants. About 80 per cent of all plants, including most field crops and many trees, harbor the fungi as an integral and normal component of their root systems. As with all fungi Arbuscular mycorrhiza also help hold soil particles together. There are about 150 species of the fungi, which may have small preferences for different soil types and environments. In general, they are all capable of colonizing roots of all susceptible plants, which was an important factor in their management (Will and Sylvia, 1990).

Arbuscular mycorrhiza extends the plant root system and the whole mycorrhiza can exploit the soil nutrients much more effectively than the plant alone. Some plant nutrients such as phosphorus and zinc, move very slowly in the soil solution. Therefore, when a plant removes these nutrients from the soil near the root, there can be a delay before they are replaced at the root surface. A zone of nutrient depletion may occur near the root and slow down plant nutrient uptake (Tilak and Annapurna, 1993).
High inorganic fertilizer applications, especially phosphorus reduce the plant’s need for Arbuscular mycorrhiza and can also reduce the fungal populations. The effect varies with the responsiveness of the crop. Wheat essentially loses its Arbuscular mycorrhiza partner when fertilizers are high, but peas, beans and many pasture legumes may still have the Arbuscular mycorrhiza and benefit from them, but to a lesser degree. Some fungicides, if they get into the soil, will reduce Arbuscular mycorrhiza populations. Most herbicides do not seem to have a direct chemical effect on Arbuscular mycorrhiza, however they do kill the plants and therefore reduce the living food source of the Arbuscular mycorrhiza fungi. Soil fumigants eliminate all soil biota, including Arbuscular mycorrhiza. This can be a problem in horticulture, especially if the crop is particularly responsive to Arbuscular mycorrhiza (Subba Rao et al., 1985).

Retaining stubble will return nutrients to the soil and the Arbuscular mycorrhiza will help to take these directly to the plants. Stubble burning kills Arbuscular mycorrhiza, especially hot burns. Some research has shown that burning stubble from a peanut crop reduced the percentage of the root length of the next crop from 72 per cent to 16 per cent. Taking into account differences in the crop growth, this translated to a reduction of Arbuscular mycorrhiza colonized roots from 12 meters per plant, to 1.5 meters per plant (Sureshkumar Singh et al., 2003).

Arbuscular mycorrhiza competes with other members of the soil biota for soil nutrients and increases the competitive ability of their host plants. They increase nodulation and nitrogen fixation in legumes by supplying the phosphate that is essential for effective nodulation. Arbuscular mycorrhiza can increase the tolerance of plants to some diseases and pests by compensating for root damage and may even have direct negative effects on the disease causing organisms themselves. Some soil animals graze on Arbuscular mycorrhiza hyphae and spores, but unless the populations are
very high and out of balance, the grazing may actually help to keep the fungi young and vigorous and release nutrients from the dead hyphae (James Kung et al., 2008).

Arbuscular mycorrhizal fungi can improve the plant growth through increased uptake of phosphorus, especially in soils of low fertility (Gerdemann, 2008) and other minerals like nitrogen, calcium, zinc, copper, manganese and iron from soil. They also induce ability to tolerate plant pathogenic microbes besides imparting some degree of moisture deficit.

The enhancement in the uptake of phosphorous and other nutrients has been demonstrated in various crops like *Liriodendron tulipifera* L. (Gray and Gerdemann, 1973); chilli (Sreeramulu and Bagyaraj, 1986); in tomato (Srihari, 1997); in potatoes (Black and Tinker, 1977); finger millet, cowpea and cotton (Bagyaraj and Manjunath, 1980) and uptake of nitrogen, calcium, zinc, copper, manganese and iron in soybean (Ross and Harper, 1970); nitrogen in soybean and maize (Kessal et al., 1985); nitrogen, calcium, zinc, copper, manganese and iron in groundnut (Krishna et al., 1982); sulphur by red clover and maize (Gray and Gerdemann, 1973).

Smith et al. (1985) reported the increase in nitrogen assimilatory enzyme like glutamine synthetase activity in clover and onion plants inoculated with *Glomus mosseae* through improved phosphorous nutrition which in turn enhanced the assimilation of ammonia in the plants. The increase in dry matter and yield has been recorded in crops like tomato (Raizhamadani et al., 1977; Ojala and Jarrell, 1980; Mohandas, 1987); peanut (Krishna and Bagyaraj, 1984); blackgram (Umadevi and Sitaramaiah, 1991); sunflower (Srihari, 1997); chilli (Sreenivasa et al., 1993; Srihari and Sreenivasa, 1993).
2.10. Interaction Between *Azospirillum* and AM fungi

Mycorrhizal fungi interact with a wide range of other soil organisms in root, in rhizosphere, mycorrhizosphere and in bulk soil. These interactions may be inhibitory or stimulatory. Some are clearly competitive others may be mutualistic. They also modify the physiology of plants including root. Similarly, other root symbionts, particularly free living nitrogen fixing bacteria can indirectly influence the behaviour of mycorrhizal fungi by changes in the host physiology (Fitter and Garbaye, 1994).

2.10.1. Enhancement of *Azospirillum* colonization by mycorrhizal fungi

Plant roots are known to exude some sugars, amino acids and growth regulators which stimulate the rhizosphere microflora (Alexander, 1961). These rhizosphere microflora interact effectively with plant roots and among themselves. The synergistic interaction between the mycorrhizal fungi and bacteria might be due to hormonal effect, nutritional relations or through physical interactions. Barea and Azcon (1982) and Sterzelezyk and Pokojska-Burdziej (1984) have reported the production of phytohormones. *viz.* cytokinins, gibberellins by mycorrhizal fungi.

Thimann (1974) had reported that growth regulators may affect the growth rate of plant organ such as the root. Alexander *et al.* (1989) have found that as mycorrhizal fungus infects the plant roots, it greatly changes the physiology of the roots and hence the nature of root exudates. This has a great effect on the development of rhizosphere microflora. Oswald and Ferchau (1968) coined the term “Mycorrhizosphere” to denote the microbial activity in soil surrounding ectomycorrhizal roots of conifers.

Enhancement of *Azorobacter* population in the root zone soil of tomato plants which were mycorrhizal has been reported earlier by Bagyaraj and Menge (1978) and other beneficial rhizosphere bacteria by Raj *et al.* (1981);
Brown and Carr (1984); Sreenivasa and Krishnaraj (1992); Krishnaraj and Sreenivasa (1990). Li and Hung (1987) isolated the nitrogen fixing organisms like *Clostridium* sp. and *Azospirillum* sp. from the surface sterilized ectomycorrhizae which indicate that organisms are probably present within the fungi. They reported that mycorrhizal fungi take up phosphorus from P deficient soil and provide as phosphate containing energy sources (ATP) that is required enormously for nitrogenase activity during nitrogen fixation by *Azospirillum* sp.

Garbaye and Bowen (1989) investigated the effect of microorganisms on the mycorrhizal colonization and also isolated them within the mantle of ectomycorrhizal fungi. Gram negative bacteria were being the largely dominant in their symbiotic stage with mutual benefit. Meyer and Linderman (1986) observed an increase in the population of specific groups of bacteria and actinomycetes populations in the rhizosphere and on rhizosplane as influenced by the development of fungi in sweet corn.

Pacovsky (1989) observed that *Azospirillum brasilense* counts per g of root was increased in the mycorrhizal (*Glomus etunicatum*) roots of sorghum. Belimov et al. (1999) also reported that mycorrhization of the plants was conducive to the better establishment of *Azospirillum lipoferum* on the rhizosphere. Klyuchnikov and Kozhevin (1990) found the increase in *Azospirillum brasilense* population in potato roots as influenced by *Glomus clarum*.

### 2.10.2. Enhancement of mycorrhization by *Azospirillum*

Many researchers have reported the influence of diazotrophic bacteria on the improved formation and development of AM fungi in the roots. Barea *et al.* (1975) suggested that the increased mycorrhizal colonization might be due to the production of plant growth substances by the rhizosphere bacteria. *Azotobacter vinelandii* and *Azotobacter beijerinckii* were observed to
produce auxins, gibberellins and cytokinins in culture (Azcon and Barea, 1975). Later, the production of plant growth substances by \textit{Azospirillum brasilense} was demonstrated \textit{in vitro} by Tien et al. (1979); Jain and Patriquin (1985) and Kapulnik et al. (1985). They hypothesized that these phytohormones causes morphological and physiological changes in the root system and enhance the plant growth.

Azcon and Barea (1978) have reported that formation of phytohormones may help in synergistic interactions between the soil microorganisms and establishing dual symbiosis with plants. Azcon et al. (1978) compared the growth and infection levels of endomycorrhizal fungi in tomato, alfalfa, lavender treated with complete bacterial cultures, cell – free supernatants and preparations of pure growth substances. The cell free supernatants or whole bacterial cultures of \textit{Pseudomonas}, \textit{Azotobacter} increased the shoot dry weight and \textit{Glomus} sp. root colonization levels as the pure plant hormones did.

Umali-Garcia et al. (1980) observed the pectinolytic activities in pure cultures of \textit{Azospirillum brasilense}, when this strain was grown in medium containing pectin. Okon (1982) suggested that \textit{Azospirillum} softens the middle lamellae of the root cortex by pectinolytic activity without causing cell collapse, thus enhancing the mineral absorption surface of cortex cells in a kind of “sponge effect”. Meyer and Linderman (1986) in \textit{Pseudomonas putida} and Will and Sylvia (1990) in \textit{Azospirillum brasilense}, have quoted the role of enzyme (pectinolytic) action produced by the bacteria, thus improving the mycorrhizal root colonization.

Pacovsky et al. (1985) reported that the extensive AMF colonization and vesicle formation in the roots also containing \textit{Azospirillum} sp. could be due to greater availability of carbohydrates to the fungal endophyte, possibly due to N-dependent increases in photosynthesis. The enhancement in
mycorrhization by the co-inoculation of *Azospirillum* had also reported by many researchers in plants like maize and rye grass (Barea *et al.*, 1983), sorghum (Pacovsky *et al.*, 1985); palmorosa (Neelima and Janardhanan, 1996) and Seaots (Will and Sylvia, 1990).

Meyer and Lindeman (1986) observed the increase in colonization of clover roots by indigenous AM fungi in presence of growth promoting rhizobacterium, *Pseudomonas putida*.

Most of the soil bacteria trigger or accelerate the germination of spores or any other dormant propagules of the fungus at pre-infection stage in soil. Azcon and Barea (1985) and Azcon (1987) studied the role of soil bacteria or their culture filtrates on the formation and development of *Glomus mosseae in vitro*. The whole bacterial culture or the metabolites, *viz.* amino acids, plant hormones and vitamins stimulated the hyphal growth at the pre-infection stages and also the new vegetative vesicles from the germinated resting spores.

Mayo *et al.* (1986) also reported the better germination of *Glomus versiformae* spores with more extensively branched hyphae in the presence of spore associated bacteria (*Pseudomonas* and *Corynebacterium*) than those from surface disinfected spores.

Few researchers have shown that bacteria can benefit the mycorrhizal formation by improving the nutrition in association, in host or in the soil. Tilak *et al.* (1989) isolated the *Azospirillum* sp. from the surface sterilized spores of vesicular-arbuscular (VA) mycorrhizal fungi (*Glomus fasciculatum, G. intraradices, G. scientillans, G. mosseae, Gigaspora gilmorei* and *Endogone dusii*). They found that the *Azospirillum* isolated from *Glomus* species had significantly more nitrogenase activity and presumed that associative diazotroph could satisfy the large amounts of
nitrogen requirement of the fungi in producing sporocarps and spores by fixing atmospheric nitrogen.

Duponsois and Garbaye (1990) reported that the bacteria associated with the ectomycorrhizal fungi (Hebeloma crustuliniforme and Paxillus involutus), produces organic acids (citric acid and malic acids) that could be utilized as carbon source by the fungi. It also metabolized the polyphenolic substances produced by the ectomycorrhiza which are toxic to the fungi itself. Tilak et al. (1990) showed that the cell free extracts of the nitrogen fixing bacteria, viz., Azotobacter chroococcum, Azospirillum brasilense and Azospirillum lipoferum enhanced the spore germination of Glomus fasciculatum.

There is evidence that, some bacteria can increase the plant susceptibility to VAM colonization. Duponsois (1992) detected the enzyme activities (endoglucanase, cellubiose hydrolase, pectate lyase and xylanase) in the pure cultures of each of several mycorrhization helper bacteria (MHBs) from the douglas fir (Laccaria laccata) and hypothesized that MHBs could soften the cell walls and middle lamella between the cells of the root cortex by producing specific enzymes and thus making fungal penetration easier.

Alarcon et al. (2002) observed the possibility of beneficial effects of phytostimulators like Azospirillum on the AM fungi (e.g., on their activity level). It was assumed that improved mycorrhization is a consequence of positive effect on root growth by Azospirillum.

2.10.3. Improvement in the nutrition and productivity of the host plant through combined inoculation of Azospirillum and AM fungi

The tripartite relationship, plant, the AM fungus and Azospirillum has been studied in detail by a number of workers because of the significance
attached in the improvement of plant growth and productivity. Kumari and Balasubramanian (1993) studied the effect of combined inoculation of AM fungi (*Glomus fasciculatum*, *Gigaspora margarita* and *Acaulospora laevis*) with *Azospirillum brasiliense* on growth and nutrient uptake of coffee seedlings grown in the nursery. They found the enhancement in growth and vigour of coffee seedlings.

Dual inoculation of maize plants with *Azospirillum brasiliense* and *Glomus mosseae*, stimulated the development of mycorrhizal fungi and produced plants of a similar size, nitrogen content and a higher phosphorous content than those supplied with N and P from the nutrient solution (Barea et al., 1983).

Subba Rao et al. (1985) reported that seed inoculation with *Azospirillum brasiliense* in conjunction with soil inoculation with *Gigaspora margarita* or *Glomus fasciculatum* produced significantly higher dry matter content of shoots, root biomass, phosphorous content and phosphorus uptake by pearl millet than the soil inoculation with AM alone. Subba Rao et al. (1985) studied the dual inoculation effects of *Azospirillum brasiliense* and various AM fungi in barley, among the different AM fungi, soil inoculation with *Glomus mosseae* and *Glomus fasciculatum* produced significantly higher dry matter production and grain yield than their corresponding controls in pot culture condition.

Konde et al. (1988) reported that simultaneous inoculation of either *Glomus* or *Gigaspora* and *Azospirillum* resulted in significant increase in fresh and dry weights and nitrogen and phosphorous uptake by shoots of bulbs of onion (*Allium cepa*) over their corresponding controls in a pot culture experiment using sterile vertic ustopepts soil. Singh et al. (1990) studied the interaction effects of *Glomus fasciculatum* and *Azospirillum brasiliense* on yields of various genotypes of wheat. They observed the
differences in AM root colonization and increased grain yield with high P$_2$O$_5$ content in the dually inoculated wheat plants grown under pot culture and field conditions.

Panwar (1991) observed that the wheat plants co-inoculated with *Azospirillium brasilense* and *Glomus fasciculatum* showed significantly higher chlorophyll content, photosynthetic rate (Ps), nitrate reductase activity (NRA) and glutamine synthetase (GS) activity and ultimately the grain yield. Veeraswamy *et al.* (1992) studied the effect of *Glomus intraradices* and *Azospirillium lipoferum* on growth of sorghum. Dual inoculation treatment resulted in significant increase in plant growth, root acid and alkaline phosphatases, uptake of P, N, Zn, Cu and Fe.

Combined inoculation of Palmarosa (*Cymbopogon martini*) with *Glomus aggregatum* and *Azospirillium brasilense* increased the growth, yield and oil content significantly over AM alone, *Azospirillium* alone or uninoculated controls (Neelima and Janardhanan, 1996). Likewise, Indi *et al.* (1990) in brinjal and Sreeramulu *et al.* (1988) in maize also observed the better growth and yield as influenced by the dual inoculation of *G. Fasciculatum* and *Azospirillium* sp.

2.11. Effect of Arbuscular mycorrhizal (AM) on agricultural crops

Arbuscular mycorrhizal (AM) fungi form symbiotic association which enhance water and nutrient transport particularly phosphorus and thereby increase growth and yield of many a crop plants (Ross and Harper, 1970; Safir Boyer and Gerdemann, 1971; Carling and Brown, 1980). Strains of *Pseudomonas fluorescens* on infection of compatible species of cereals participate in the symbiotic association, root nodulation leading to more nitrogen fixation which increases the growth and yield (Dixon and Wheeler, 1986). In recent years, the effect of combined inoculation with Arbuscular mycorrhizal fungi and *Pseudomonas fluorescens* have been reported to
further increases the growth and yield of some crops including maize (Young et al., 1988). The tripartite association of symbiotic Arbuscular mycorrhizal fungi, *Pseudomonas fluorescens* and root nodule bacteria *Bradyrhizobium japonicum* was investigated to find its effect on the promotion of growth and yield of maize.

According to a fossil record, Arbuscular mycorrhizal fungi were infecting in the roots of *Aglaophyton major*, an early devonian land plant, at least 400 million years ago (Remy et al., 1994). The early devonian has been known to be the period when plants invaded the land, and it will be of great interest to imagine how the plants invaded the land where there was a severe situation for them with the help of Arbuscular mycorrhizal fungi.

Arbuscular mycorrhizal fungi stimulate phosphorous uptake of plants from the soil, a great deal of phosphorous in the soil inhibits Arbuscular mycorrhizal growth. A concentration of over 50 ppm of PO₄ in the soil has been reported to inhibit severely Arbuscular mycorrhizal colonization. However, the application of insoluble phosphate, such as rock phosphate (Graham and Timmer, 1985), calcium phosphate and bone powder was effective to maintain Arbuscular mycorrhizal fungi. An additional advantage of insoluble phosphate was long term availability of P, compared to soluble phosphate.

Microbe - microbe interactions are crucial to understand the dynamic processes characteristic of rhizosphere establishment and maintenance. Mycorrhizal fungi and certain other soil microorganisms are known to regulate mycorrhizal formation and function. Conversely, mycorrhizae affect the establishment of rhizosphere populations. Microbial population (root associated mycoflora) in soil concentrate around plant roots stimulated by root exudates supplied by the plants (Bowen, 1980; Curl and Truelove, 1986). The population dynamics of microorganisms determines
the competitive interactions at the root soil interface by the limited carbon resources released by the plants. On the other hand, added organic matter results in a flush of microorganisms (Russell, 1973).

Sureshkumar Singh et al. (2003) studied the regeneration pattern and species diversity of Arbuscular mycorrhizal (AM) fungi in shifting cultivated abandoned land (jhum fallow) and natural forest soils. The jhum fallow contained lower VAM fungal population and number of species than the natural forest. A total of 44 Arbuscular mycorrhizal species belonging to six genera namely *Acabospora*, *Enterophospora*, *Gigaspora*, *Glomus*, *Sclerocystis* and *Scutellospora* were recorded from soils of jhum fallow and natural forest sites. Ten species of Arbuscular mycorrhizal fungi were found eliminated from the jhum fallow revealing that the shifting cultivation in the humid tropical soils causes reduction of Arbuscular mycorrhizal fungal species.

Most species of plants are capable of associating with fungi of a single family, *Endogonaceae*, to form Arbuscular mycorrhizal (Gerdemann, 1968). This symbiotic association of the endomycorrhizal fungi and roots of plants are beneficial with the exchange of nutrients between the symbionts. Mycorrhizal fungi are benefited with carbon substrates from plants and in turn the plants are provided with nutrients especially phosphorous compounds from soil solution through the hyphal network of the fungi apart from increased absorptive surface area of the roots (Janos, 1980; Lohananchan, 2000; Ravarkar et al., 2000).

Ghosh et al. (2004) studied the fluctuation in mycoflora population under low (25 per cent), medium (50 per cent) and waterlogged moisture conditions by treating rice with Arbuscular mycorrhizal + *Azolla* combinations and singly with Arbuscular mycorrhizal and *Azolla*. Variations were recorded as number of CFU g⁻¹ soil × 10⁴ using rhizosphere soil.
Fungal population was found to be increased and well maintained both in medium moisture condition and in Arbuscular mycorrhizal + *Azolla* combined treatment. This condition was found to be congenial for both mycorrhizal colonization and leaf phosphate accumulation in rice.

Solaiman and Hirata (2004) examined the effect of Arbuscular mycorrhizal fungi inoculation at the nursery stage on the growth and nutrient acquisition of wetland rice. Seedlings were grown on gamma ray sterilized paddy soil in two types of nurseries, namely dry nursery and wet nursery, with or without arbuscular mycorrhizal fungi Arbuscular mycorrhizal inoculation which was a mixture of indigenous Arbuscular mycorrhizal (*Glomus* sp.) spores collected from the paddy field. Five to six week old seedlings were transplanted to the unsterilized soil under field and pot, respectively. Mycorrhizal seedlings had higher shoot biomass under both nursery conditions 5 weeks after sowing.

Mycorrhizal colonization and sporulation were 2 to 3 times higher in the dry nursery than the wet nursery at the transplanting stage. Mycorrhizal colonization of plants inoculated in the nursery remained higher than those not inoculated under both field and pot conditions. Sporulation after transplanting to field conditions was about 10 times higher than in the pot. Inoculated plants produced higher biomass at maturity under field conditions and the grain yield was 14 – 21 per cent higher than those not inoculated. Conversely, grain yield and shoot biomass were not significantly influenced by Arbuscular mycorrhizal colonization under pot conditions. For plants originating from the dry nursery, N, P, Zn and Cu concentrations of field grown plants at harvest were significantly increased by pre-inoculation with Arbuscular mycorrhizal over those left uninoculated.

Secilia and Bagyaraj (2004) tested the response of the wetland rice cultivar to inoculation with ten Arbuscular mycorrhizal (AM) fungi in a pot
experiment under flooded conditions in order to select the most efficient mycorrhizal fungi to inoculate the rice nursery. A sandy clay loam soil was used as the substrate, fertilized with the recommended N and K levels (100 kg N ha⁻¹ as ammonium sulphate and 50 kg K ha⁻¹ as muriate of potash) and half the recommended level of P (25 kg ha⁻¹ as super phosphate). The inoculation was made into dry nursery beds and the beds were flooded when the seedlings were about 25 cm high, in 15 days. Twenty-eight-day old seedlings were transferred to pots filled with well puddled soil flooded with 5 cm of standing water. Based on the increase in grain yield and total biomass, *Glomus intraradices* and *Acaulospora* sp. were considered efficient and suitable for inoculation into rice nurseries.

Song Yung Chen (2005) carried out experiments to study the effects of phosphorous sources on phosphatase activity of rhizosphere and mycorrhizosphere of red clover inoculated with *Glomus mosseae* and cultural system with three compartments was applied. The plants were harvested after 8 weeks, and measured for dry weight and phosphorous contents as well as soil phosphatase activity of root and hyphal compartment. The results showed that acid phosphatase activity was higher than alkaline phosphatase activity and both of them were increased slightly after inoculation, especially supplied with organic phosphorus. Acid phosphatase activity was higher than alkaline phosphatase in the mycorrhizosphere of all inoculated treatments. The dry weight, phosphorous content and total phosphorous uptake of the plants increased significantly after inoculating mycorrhizal fungus. The amount of phosphorous uptake by hyphae accounted for 43.1 per cent of total phosphorous uptake of plants under applying KH₂PO₄, while the value was 60.8 per cent under applying sodium phytate.
James Kung et al. (2008) studied the influence of Arbuscular mycorrhiza fungi inoculation on coppicing ability and drought resistance of *Senna spectabilis* in a screen house experiment. The result obtained indicates the dependence of *Senna spectabilis* on mycorrhizal symbiosis. Under well watered conditions, Arbuscular mycorrhiza (AM) inoculation increased coppicing biomass production of *Senna spectabilis* by 26.9 per cent, while under water stressed conditions, coppice biomass production increased by 317 per cent. Analysis of variance revealed that interaction between the mycorrhizal fungi and water stress was highly significant. Inoculating *Senna spectabilis* with Arbuscular mycorrhizal improved its drought resistance. Under drought conditions, inoculating *Senna spectabilis* increased total shoot length by 100 per cent root collar diameter by 74 per cent shoot dry weight by 435 per cent root dry weight by 397 per cent and plant leaves number by 105 per cent. Inoculated plants had more leaf water content than non inoculated plants. Inoculated *Senna spectabilis* plants took more days to show signs of drought stress. The better growth responses of mycorrhizal plants were attributed to higher nutrients uptake and higher moisture absorption. Arbuscular mycorrhiza (AM) inoculation has a high potential in water stressed environment in maintaining water relationship.

Pandey and Baink (2009) conducted preliminary experiments in glass house to study the effectiveness of Arbuscular mycorrhizal (AM) fungi on the medicinal plant *Aloe vera*. The *Aloe vera* plants were grown with Arbuscular mycorrhizal fungi inoculation in polythene bags showed an increase in plant growth (leaf, stem and root dry biomass), root colonization, phosphorus, nitrogen, sugar and barbaloin content over those grown without inoculation. *Glomus mossae* was found to be the best Arbuscular mycorrhizal symbionts for inoculating *Aloe vera*. An orthogonal experimental 9 design L (3) in glass house was conducted to investigate the effect of *Glomus mossae* (5 or 10 g/plant), *Azotobacter* sp. 41 (10^3 and
$10^6$ cfu ml$^{-1}$), rock phosphate (50 and 100 mg/ kg soil) and soil type (per cent sand in garden soil v/v) (50 and 100 per cent v/v) on *Aloe vera* plant growth and barbaloin content (mg plant$^{-1}$). Inoculation by both *Glomus mossae* and *Azotobacter* sp. 41 showed increase in Barbaloin content plant$^{-1}$.

Viswanathan Guru *et al.* (2011) assessed the influence of co-inoculation of AM fungi and *Azospirillum* on the growth characteristics, quality, nutrient contents and yield of tomato crop varieties, pot culture experiments were conducted. The plant growth effects, number of fruits per plant, yield per plant, nutrient contents, available soil N and P, per cent root colonization and the quality of fruits were measured for the singly inoculated, co-inoculated and un-inoculated control crops. Overall, the crops that were co-inoculated with the bio-inoculants expressed good growth, nutritional characteristics, better yield and quality fruits.

Sandeepkumar *et al.* (2011) conducted an experiment in soils which are low in available phosphorous and with a high indigenous AM fungal population or exotic AM fungal species and other beneficial microorganisms. Such conditions usually promote positive responses in the experimental plants. Significantly increased growth was observed in plants with triple inoculation of AM fungi, *Azotobacter* and PSB than dual inoculation of *Glomus fasciculatum* + *Azotobacter* and *Glomus fasciculatum* + PSB over uninoculated control plants.

Sivakumar and Thamizhiniyan (2012) investigated the influence of AM fungi and *Azospirillum* on nutritional potential of tomato (*Lycopersicon esculentum*). They conducted as field experiment at Sivapuri Village, Annamalai Nagar, Tamil Nadu, India. The influence of VAM and *Azospirillum* on the tomato plant *Lycopersicon esculentum* Mill. The leaf nutrients and fruit nutrients were higher in VAM + *Azospirillum* group. It was clear that the *Azospirillum* along with AM fungi showed better result
when compared to control. The main advantage of this association was it does not pollute the soil and also does not show any negative effect to environment and human health.

Kanchana et al. (2013) collected the soil samples from ten different locations in Cuddalore district, Tamil Nadu. The Azospirillum isolates were screened for their efficiency by determining the ability to produce phytohormone Indole acetic acid (IAA), Nitrogen fixation and phosphorus solubilization. The isolates of Azospirillum were designated as Azs-1 to Azs-10. The highest IAA production was recorded by the isolate Azs-2 obtained from Sivapuri soil. The nitrogen fixation of Azospirillum isolate ranged from 9.8 ± 0.3 to 16.9 ± 0.5 μg of nitrogen g of malate utilized. Interestingly, 1 the maximum nitrogen fixation 16.9 ± 0.5 kg of N g 1 of malate was also recorded by the isolate Azs-2.

Tamwar et al. (2013) studied the effect of two arbuscular mycorrhizal fungi [G. mosseae (G) and A. laevis (A)] with P. fluorescence (Pf) in the presence of super phosphate (P) fertilization on growth and yield of bell pepper (Capsicum annuum var. California Wonder) in pots under greenhouse conditions, in a completely randomized design with four levels of phosphorus fertilizer [F0–without P, F1–0.200g pot⁻¹ (half of the recommended dose), F3–0.400g pot⁻¹ (recommended dose) and F4–0.800g pot⁻¹ (double the recommended dose)] having six different combinations of bioinoculants. Inoculation of bioinoculants with F₁ increased plant growth and nutrition to an acceptable level with AM fungi in combination with P. fluorescens. Application of higher dose of P fertilizer markedly decreased all the growth parameters. The prevalence of AM colonization was highest in G+A+Pf with F₁. Similarly, highest yield was recorded for the treatment involving multi inoculation of G+A+Pf in the treatment of F₁ followed by dual inoculation of G+Pf in F₀ plants. Thus, this finding suggests the
application of efficient bioinoculants (G+A+Pf) along with right dose of P fertilizer (half of the recommended P) during seedling transplantation to increase overall growth and yield performance of bell pepper and could be considered as a sustainable substitute to higher phosphorus fertilizer for bell pepper cultivation.