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Review

A review on the role of *Azospirillum* in the yield improvement of non leguminous crops

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Rise in human population always demands a rapid and sustainable increase in cereal production. As a result nitrogenous fertilizers were used constantly in excess, which resulted in a number of problems such as green house emissions (particularly N_2O) and leaching to ground-water. Moreover they are expensive. So long term sustainability in agriculture can only be obtained with the use of low cost fertilizer which should also be ecologically safe. In this regard biological nitrogen fixation by microbes, that is, biofertilizer, plays an active role helping in better maintenance of crop nutrient as well as soil health. *Azospirillum*, an associative symbiotic nitrogen fixing bacterium has a higher nitrogen fixing potential in non-legumes in comparison to other nitrogen fixing bacterium, by the formation of *para* nodules. However further investigation is needed to find possible avenues for the exploitation of this bacterium. The current review emphasizes the central issues of *Azospirillum* and its application either alone or in combination with other plant growth promoting rhizobacteria for the benefit of the non leguminous crops.

Key words: *Azospirillum*, non legumes, BNF, *para*-nodules, inoculation, rhizosphere.

INTRODUCTION

Non leguminous like rice, maize and wheat belonging to the family Poaceae are important to the millions of farmers who grow these crops, to many landless workers who derive income from working on these farms and to the billions of consumers of these crops around the world. Rapid increase in world population indicates the need for increased production of the same. According to Food and Agricultural Organization (FAO) world cereal production in 2008 was forecasted to increase 2.6% to a record 2,164 million tons. But with the increase in cultivation of cereals, use of chemical fertilizers is also increasing simultaneously as it can rapidly give more reliable boost to crop yield. For example, in an experiment in UK the application of inorganic fertilizer @ 192 $kgNha^{-1}$ provided an additional 5.72 tonnes of wheat compared to a corresponding plot that had no additional N added. This represented to a 485% increase in revenue from the same area of land even after additional processing costs and the price of the inorganic N were accounted for (Jenkinson, 2001). Approximately 65% of the applied mineral N is lost from the plant-soil system through gaseous emissions, run off, erosion and leaching. Environmental impact of this loss ranges from green house effect, diminishing stratospheric ozone and acid rain to changes in the global N cycle and nitrate pollution of surface and ground water (Rejesus and Hornbaker, 1999). Moreover the production of inorganic N by the Haber–Bosch process generates huge amount of CO_2, between 0.7-1.0 tonnes per tonnes of Ammonia. At the same time due to unavailability of fossil fuels, the price of chemical fertilizers is also increasing rapidly since inorganic fertilizers are derived from fossil fuels particularly Natural gas. There are also problem of losses of fertilizer after application through leaching, volatilization, and through denitrification. Overall, effects

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of these problems requires more concentration on greater access to inexpensive biofertilizer technologies, as they are ecologically sound and their application could help to minimize the global warming as well as to reduce the fertilizer input in farming practices. If Biological Nitrogen Fixation (BNF) system could be assembled in the non–legumes plants it could increase the potential for nitrogen supply because fixed N would be available to the plants directly, with little or no loss. Thus a significant reduction in the relative use of fertilizer N can be achieved if atmospheric N is made available to non-legumes directly through an effective associative system with some of the characteristics of legume symbiosis. Since the rediscovery of *Azospirillum* by Dobereiner and her collaborators in the 1970s, the species *Azospirillum* has gained the reputation of being the most studied plant-associative bacterium as it fixes atmospheric nitrogen and produces phytohormones (Tien et al., 1979). Apart from direct agricultural application, *Azospirillum* is an excellent model for genetic studies of plant-associative bacteria in general. The largest portion of *Azospirillum* literature consists of genetic studies of almost all aspects of the bacterium and its association with plants. As the most researched associative bacterium, Azospirilla has become a corner stone of rhizosphere research unrelated to its questionable field application (Bashan and Holguin, 1997).

Apart from being a general plant colonizer (Bashan et al., 2004), *Azospirillum* is remarkably versatile. *Azospirillum* not only fixes atmospheric N (Dobereiner and Day, 1976), but can also mineralize nutrients from the soil, sequester Fe, survive under harsh environmental conditions, and support beneficial mycorrhizal-plant associations (Bashan et al., 2004). In addition, *Azospirillum* can help plants minimize the negative effects of abiotic stresses. Because *Azospirillum* is the most studied PGPB, excluding rhizobia, and reached commercialization in several countries, including Argentina, Mexico, India, Italy, and France (Diaz-Zorita and Fernandez-Canigia, 2009; Hartmann and Bashan, 2009), considerable knowledge has been accumulated during the last three decades. In this review, the recent advances on how *Azospirillum* promotes the growth of non leguminous plants are highlighted. Furthermore, the role of *Azospirillum* used either alone or in combination with other Plant Growth Promoting Rhizobacteria (PGPR) affecting the performance of non legumes in different ecological niches is discussed.

Taxonomy, general characters and host range

Among various microbial species, *Azospirillum,* is the starting point of most ongoing biological nitrogen fixation program with non legumes plants worldwide. They are known as plant growth-promoting bacteria (PGPB) because of their ability to stimulate growth. Beijerinck (1925) first isolated this bacterium from sandy soil and named it as *Spirillum lipoferum* which was renamed as *Azospirillum lipoferum* by Tarrand et al. (1978). *Azospirillum* species is included in α subclass of proteobacteria belonging to the IV rRNA superfamily (Xia et al., 1994).

Bacteria belonging to the genus *Azospirillum* are plump, slightly curved and straight rods, about 1.0µm in diameter and 2.1-3.8 µm in length, often with pointed ends. They are highly motile. *A. brasilense, A. lipoferum and A. irakense* display a mixed pattern of flagellation (Hall and Krieg, 1984; Moens et al., 1995). One polar flagellum is synthesized during growth in liquid medium and is primarily used for swimming. Additional lateral flagella are induced during growth on solidified media and are responsible for swarming of the bacteria over solid surfaces. *A. halopraeferens and A.amazonense* only displays the polar flagellum (Moens et al., 1995). Motility offers the bacterium the advantage of moving towards favorable nutrient conditions. *Azospirilla* exhibit positive chemotaxis towards organic acids, sugars, amino acids, aromatic compounds as well as towards root exudates (Heinrich and Hess, 1985). Earlier data claims that Azospirilla shows specificity for certain cereals. However data published in recent years showed that *Azospirillum* had no preference for crop plants or weeds, or for annual or perennial plants and can be applied successfully to plants that have no previous history of *Azospirillum* in their roots. Thus, it is evident that *Azospirillum* is a great root colonizer and not a plant-specific bacterium (Bashan and Holguin, 1997). Since then, *Azospirillum* has been isolated from the roots of numerous wild and cultivated grasses, cereals and legumes and from tropical, subtropical and temperate soils world-wide (Bashan and Holguin, 1997).

Azospirillum-soil interaction

Azospirillum species have always been identified mainly as rhizosphere bacteria. They proliferate in the rhizosphere of a few perennials and most annuals (Bashan and Holguin, 1997) but this bacteria can even survive in the absence of their host, owing to the presence of some special physiological mechanisms which are cyst formation (Bashan et al., 1991), flock formation (Neyra et al., 1995), production of melanin (Givaudan et al., 1993), and poly-β-hydroxybutyrate (PHB) (Okon and Itzigsohn, 1992), polysaccharide synthesis (Del Gallo and Idaegi, 1990) and protection inside ectomycorrhizal fungal spores (Li and Catellano, 1987). However though *Azospirillum* species are known as typical rhizosphere bacteria, no important phase in the bulk soil has been found for this genus (Bashan, 1999). Several factors are responsible for the general survival of *Azospirillum* in soil which is related mainly to the geographic origin of the soil and not to the prevailing environment condition. The

adsorption of introduced bacteria depends mainly on the physicochemical composition of the soil particles and to a lesser extent, on the bacterial species present or the bacterial growth condition prior to inoculation. Theoretically bacteria, clays, and organic matter particles possess a similar net negative surface charge that prevents contacts between them but adsorption of bacteria to soil do occur which may be because clay particle may possess positively charged edges to which bacteria can adsorb. Adsorption of microorganisms to soil enhances by a decrease in soil pH which increase the positive charge density on the edge of clay particles or by flooding the soil (Bashan and Levanony, 1989). An additional soil variable that may affect adsorption of *Azospirillum* spp. is the cation exchange capacity (CEC) of the soil. Adsorbed cell percentage increase with the increase in CEC (Govindarajan and Purushothamam, 1989) and finally adsorption may also be affected by soil redox potential which directly affects N_2 fixation by *Azospirillum* spp. In sand a different mechanism of adsorption operates. Incubation *of A. brasilence* Cd in quartz sand resulted in its attachment to sand particles by protein bridging which prevents the cell from being washed away. However, only 56% of the applied bacteria are adsorbed (Bashan and Levanony, 1988). Early observation indicates that Azospirilla require neutral pH for abundant occurrence (Dobereiner and Day, 1976; Dobereiner et al., 1976), while their presence was observed to be 70% in soil with pH between 5.8 and 6.2 and 40% in soils with pH between 4.8 and 5.1 (Dobereiner, 1978). Roots however seem to provide suitable environment for Azospirilla in acid soil also.

Azospirillum-plant relationship

Several physiological, environmental, nutritional and chemical factors enhanced or suppressed *Azospirillum* species attachment to the roots (Umali-Garcia et al., 1980; Bashan and Levanony, 1989). Lectin binding has been suggested as a possible mechanism (Umali-Garcia et al., 1980) and recently it was speculated that agglutinins may be located in the fibrilar material, helping cell anchorage (Bashan and Levanony, 1988). It involves the following events: (i) the bacterium is first chemotactically attracted by the root exudates, both specifically (by a proteic compound and by selective carbon compounds) and un-specifically, (ii) it then loosely adheres to the root surface by flagella and some glycocalyx compounds (phase 1 adhesion). During this step, agglutination can be induced by plant lectins, (iii) after attachment, an exchange of message occur between plant and bacterium (are flavones/flavonoids involved, like in *Rhizobium*-legume symbiosis?) It could be the binding of other lectins and polysaccharides (iv) as a result of signal exchange, cellulose fibrils are produced by *Azospirillum*, which anchor the bacteria more tightly to the root surface (phase 2 adhesion) leading to (v) the viable and fully established association. Consequently, production of plant growth promoting substances by the bacterium and a stimulation of the endogenous plant hormones production takes place (Fallik et al., 1994).

Facilitation of plant growth

Principal mechanism as to how exactly *Azospirillum* facilitate the growth of plant is undetermined. However, there are several modes of action other than BNF such as phytohormone production (Perrig et al., 2007) and bacterial nitrate reductase activity in roots which increases nitrate accumulation in inoculated plants. Instead, the most accepted hypothesis postulates that a sum of events accounts for *Azospirillum* mediated growth promotion activity which are briefly discussed.

Nitrogen fixation

Azospirillum can exist freely or in symbiosis and in either case entraps atmospheric nitrogen and converts the unreactive nitrogen to NH3, a form that is readily utilize by plants, this process is termed as biological nitrogen fixation (BNF) and is catalyze by the oxygen sensitive enzyme nitrogenase present within the bacteria by the following reaction;

$$N_2 + 8H + 8e\text{-} + 16\ ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

The ability of an endophyte to fix atmospheric nitrogen within a host has been proved using different approaches: acetylene reduction assay, ^{15}N isotope dilution (Van Berkum and Bohloo, 1980). However, conclusive proof that plants derive some of their N from the atmosphere came from the use of isotopic $^{15}N_2$ incorporation and ^{15}N dilution techniques. These subjects have been adequately reviewed (Boddey, 1987; Boddey and Dobereiener, 1988). *Azospirillum* has been proposed as the major organisms responsible for the nitrogenase activity in different plants. According to a detail survey it has been found that nitrogen fixation by the *Azospirillum* to the plants is minimal and range from 5-18% of total plant increase (Bermner et al., 1995) and the observed plant growth promoting effects can be attributed to other mechanisms like phytohormone production and displacement of pathogens. The nitrogenase activity of *Azospirillum* has been found to increase when grown in mixed cultures with other bacteria, even if they come from completely different habitats (Khammas and Kaiser, 1992; Holguin and Bashan, 1996). Apparently some mixed cultures provide conditions more suitable for N_2-fixation than those present in pure culture. An example for an extremely unlikely association is the mixed culture of *A. brasilense* Cd and the non N_2-fixing, marine

Mangrove rhizospher bacterium *Staphylococcus* sp. that increases the N_2-fixation of the former. The effect was stronger when diluted *Staphylococcus* supertananat was added to *A.brasilense* culture and was partially due to release of aspartic acid from the *Staphylococcus sp* cells (Holguin and Bashan 1996). N_2-fixation was the original proposed mechanism by which *Azospirillum* effects plant growth. Evidence that N_2-fixation contributes to the N balance of plants is based on the common observation of an increase in the nitrogenous activity within inoculated roots (Kapulnik et al., 1981; Okon et al., 1983; Yahalom et al., 1984). This well documented enzymatic activity is of sufficient magnitude to account for the increase in total N-yield of inoculated plants if the entire fix N is incorporated into the plants (Sarig et al., 1984; Mertens and Hess, 1984).

Growth substrates and their influence on nitrogen fixation

Organic acids and sugars

The utilization of substrates available as plant cell constituents, mucilages and in root exudates is a prerequisite for growth in the rhizosphere, colonization of the root and finally, nitrogen fixation in association with the root. The chemotaxis towards components of root exudates and mucilage (Heinrich and Hess, 1985; Reinhold et al., 1985; Mandimba et al., 1986) and the utilization of organic acids and sugars by *Azospirillum* is well documented (Magalhaes et al., 1983; Reinhold et al, 1987). There are species-speciic diferences in the utilize-tion of sugars; thus *A. lipoferum* readily utilizes glucose and *A. amazonense*, sucrose, whereas *A. brasilense* and *A. halopraeferens* cannot grow on these substrates efficiently. The physiological basis for these differentiating characteristics is the abilities to take up mono- and disaccharides and utilize them along different pathways (Martinez-Drets et al., 1984; Martinez-Drets et al., 1985).

Amino acids

A. lipoferum and *A. amazonense* readily used many amino acids as the sole sources of carbon, nitrogen and energy. Nitrogen fixation measured in the presence of malate or sucrose was drastically inhibited at high concentrations of amino acids. At low concentration of glutamate, nitrogen fixation in *A. amazonense* was slightly stimulated. In contrast, *A. brasilense* and *A. halopraeferens* grew poorly or not at all with amino acids as sole source of carbon, nitrogen and energy (exception: alanine), and nitrogen fixation was not inhibited at even high concentrations of amino acids, for example, glutamate. Accordingly, glutamate is a good nitrogen source for *A. lipoferum* and *A. amazonense*, but a poor nitrogen source for *A. brasilense* and *A. halopraeferens* (Hartmann et al., 1988). The physiological basis for the different efficiency of the utilization of glutamate is probably the different activities for glutamate uptake, glutamate dehydrogenase and glutamate transaminases (Hartmann et al., 1988). The ability of *Azospirillum* spp. to grow on amino acids as sole sources of carbon and nitrogen may be helpful as additional taxonomic characteristics and to isolate certain species speciically (for example, *A. lipoferum* with histidine). This ability may also be relevant for their growth behaviour in the rhizosphere of particular plants and for the establishment of associative nitrogen fixation

Polymer-substrates and C_1 – compounds

Azospirillum can grow and fix nitrogen using polymer substrates, such as straw and xylan (Halsall et al., 1985) and hemicellulose (Ladha et al., 1986). In co-culture with cellulose degrading bacteria, *Azospirillum* can fix nitrogen (Halsall and Gibson, 1985). The ability for autotrophic growth is widely distributed among *Azospirillum* (Malik and Schlegel, 1981; Tilak et al., 1986). *Azospirillum spp*., including the recently described *A. halopraeferens*, is able to grow on methanol or other C_1 -compounds and fix nitrogen (Sampaio et al., 1982). *Azospirillum* may also be able to use the methyl groups of pectin, a major constituent of plant cell walls. Cell-wall degrading enzymes are present in *Azospirillum* at rather low activities (Tien et al., 1981).

Regulation of nitrogenase activity by ammonium and oxygen

Ammonium

After the addition of very low concentrations of ammonium, nitrogen fixation is rapidly and reversibly inhibited in *Azospirillum* spp. (Gallori and Bazzicalupo, 1985; Hartmann et al., 1986). A covalent modification of the nitrogenase reductase of *A. brasilense* and *A. lipoferum* was demonstrated using the quick filtration and extraction procedure (Kanemoto and Ludden, 1984) in combination with the immuno-blotting technique (Hartmann et al., 1986). The modification resembles the situation in the photosynthetic bacterium *Rhodospirillum rubrum*, where a 'switch off' of nitrogenase activity is caused by an ADP-ribosylation of nitrogenase reductase (Pope et al., 1985). In *A. amazonense* (Song et al., 1985; Hartmann et al., 1986) and *Herbaspirillum seropedicae* no evidence for an involvement of covalent modification in the 'ammonium switch off' was apparent. Probably, the less complete inhibitory effect is mediated by a noncovalent inhibitory mechanism in these bacteria. The rapid inhibition of nitrogenase activity by ammonium and

other nitrogen compounds is widely distributed in N_2-fixing bacteria (Turpin et al., 1984; Kush et al., 1985; Heda and Madigan, 1986).

However, the appearance of a covalently modified nitrogenase reductase during 'switch off' has clearly been demonstrated only in a few species (Gotto and Yoch, 1985; Hartmann et al., 1986). The involvement of an activating (Saari et al., 1984) and inactivating enzyme (Lowery et al., 1986) in the regulation of nitrogenase reductase probably improves the fine tuning and efficiency of nitrogenase regulation. *A. brasilense* and *A. lipoferum* have an activating enzyme, which activates modified nitrogenase reductase of *Rhodospirillum rubrum* (Ludden and Burris, 1976; Ludden et al., 1978). The regulatory system of nitrogenase in *R. rubrum*, a close relative to *Azospirillum*, appears to be very similar to *A. brasilense* and *A. lipoferum.*

Oxygen

After a shift to anaerobic conditions, nitrogen fixation of *A. brasilense* and *A. lipoferum* was inhibited and simultaneously the nitrogenase reductase was modified. However, during the rapid inhibition of nitrogenase activity by high oxygen levels, no covalent modification occurred. The decrease of nitrogenase activity during anaerobiosis was not accompanied by an increase of the glutamine pool, as in the 'ammonium switch off', and no alteration in the activity of glutamine synthetase occurred (Hartmann and Burris, 1987). Therefore, a signal independent of the nitrogen metabolism can also trigger the inactivating enzyme which catalyzes the covalent modification of nitrogenase. A decrease in the oxoglutarate level could also provide the signal, because this would raise the glutamine/oxoglutarate ratio, which is of general importance in metabolic control of nitrogen metabolism.

Release of nitrogen compounds by Azospirillum

At low levels of glutamate or aspartate and small amounts of ammonium are released from N_2-fixing cultures of *A. brasilense* and *A. amazonense* (Hartmann et al., 1988). Glutamate and aspartate, or metabolities derived from it, might reduce the activities of ammonium assimilatory enzymes and ammonium uptake and foster ammonium release. A release of nitrogen compounds in laboratory batch cultures of *A. lipoferum* (Volpon et al., 1981), *A. amazonense* and *A. halopraeferens* (Hurek et al., 1987) has been described. Energy starved cultures of *Azospirillum* release small amounts of ammonium due to an inacti-vation of the ammonium permease and a decrease in the activities of ammonium assimilatory enzymes (Hartmann et al., 1984). After the addition of malate, ammonium uptake is rapidly reactivated and ammonium is taken up again. This mechanism of nitrogen release might operate in rhizocoenoses, when the energy supply is transiently reduced due to environmental effects on the plant (Whiting et al., 1986) or at later growth stages, when the root becomes a minor sink for photosynthates (Curl and Truelove, 1986).

Ammonium release and constitutive N_2-fixation in mutants

Glutamine auxotrophic mutants of *A. brasilense*, defective in glutamine synthetase (Gauthier and Elmerich, 1977) or glutamate synthase (Bani et al., 1980), release ammonium under N_2-fixing conditions; these mutants have no ammonium uptake activity (Hartmann et al., 1984). Prototrophic mutants of *A. brasilense* which fix nitrogen constitutively were isolated (Fischer et al., 1986). In prototrophic mutants of *A. brasilense* resistant to methionine sulphoximine (MSX), constitutive N_2-fixation occurred but no ammonium release was observed (Hartmann, 1982; Hartmann et al., 1983). So it was speculated that mechanisms for the release of nitrogen compounds are present in bacteria from N_2-fixing associations, but this property is under the physiological control of plant factors (Hartmann, 1988).

Inhibition of nitrogen fixation by oxygen and oxygen protective mechanisms

Species and strain specific differences in oxygen tolerance

All *Azospirillum* species are characterized as micro-aerobically nitrogen fixing bacteria (Krieg and Dobereiner, 1984; Okon et al., 1977). The narrow range of oxygen tolerance obviously limits nitrogen fixation in the association with roots (Zuberer and Alexander, 1986). A comparison of the oxygen tolerance of nitrogen fixation in a well mixed chamber (Hochman and Burris, 1981), which allowed simultaneous measurements of dissolved oxygen, respiration activity and acetylene reduction, revealed an increased oxygen tolerance in the order *A. brasilence* Sp7, *A. lipoferum* SpRG20a and *A. amazonense* Y1 (Hartmann et al., 1985). Cells growing with ammonium chloride as nitrogen source were centrifuged and resuspended in nitrogen free minimal medium. Derepression of nitrogen fixation was examined at different constant dissolved oxygen concentrations in a rapidly stirred chamber equipped with an oxygen electrode (Hartmann et al., 1986). After the acetylene reduction activity reached a constant rate the speific activity was determined. For comparison reasons the highest acetylene reduction rate in each train was set to 100%. The lag period until the

acetylene reduction rate reached a fairly constant rate was considerably prolonged at increasing dissolved oxygen concentrations. The dissolved oxygen concentration in equilibrium with 1 kPa oxygen equals about 12 µ*M.*

Oxygen protection by carotenoids

Carotenoids contribute to the oxygen tolerance due to their ability to quench radical reactions which are initiated by toxic oxygen metabolites, thereby preventing auto-oxidation reactions (Burton and Ingold, 1984). Carotenoid over producing mutants, obtained from the slightly pink wild type *A. brasilense* Sp7, grew and fixed nitrogen at oxygen stress conditions, whereas the wild type failed to grow (Hartmann et al., 1983). In oxygen controlled, nitrogen-free continuous cultures an improved nitrogen fixation activity occurred due to carotenoids at oxygen stress conditions (12 µM dissolved oxygen), although growth and nitrogen fixation was greatly inhibited. It is concluded that a high level of carotenoids do not extend the optimum for nitrogen fixation to higher oxygen levels, but provide some limited protection against oxygen damage.

Reversible 'oxygen switch off'

A rapid, and partially reversible, inhibition of nitrogenase activity by oxygen was observed in *Azospirillum* spp. (Hartmann and Burris, 1987). Goldberg et al. (1987) suggested that at high oxygen levels the limited pool of electrons is diverted towards respiration, thus leaving the nitrogenase without a suficient supply of reduction equivalents.

Respiratory protection

A comparison of the activities of citrate synthase and other TCA-cycle enzymes of *Azospirillum* (Martinez-Drets et al., 1984) with *Azotobacter* (Ramos and Robson, 1985) reveals that respiration protection of *Azospirillum* may indeed be limited by the supply of reduction equivalents. Therefore, attempts to improve oxygen tolerance of nitrogen fixation should focus on an improvement of respiratory capacity, so that enough electrons are avaiable to support high respiration rates and high nitrogenase activity simultaneously. This may in part be accomplished by providing *Azospirillum* with an optimum iron supply, because iron is an important constituent in many of the enzymes involved.

Osmotolerance and osmoregulatory properties

Metabolic activities of microorganisms are greatly influenced by the availability of water (Brown, 1976). Soil bacteria have to cope with water stress in salt affected soils or when soil becomes dry. Low water potentials frequently occur in the rhizosphere of plants growing in a dry climate, because of the high water need of the transpiring plant. In microorganisms, which are able to adapt to changing water potentials in the environment, compatible solutes like amino acids, betaines and sugars play an important role (Imhoff, 1986; Ken et al., 1986). The intracellular accumulation of these solutes prevents water loss from the cell and is compatible with or even preserves enzymatic functions. In drought or osmotic stress conditions various plants, including grasses, accumulate betaines and/or proline (Wyn Jones and Storey, 1981). Probably these substances are available for microorganisms in the rhizosphere.

In *Azospirillum*, osmotolerance is a species-speciic character and declines in the order *A. halopraeferens, A. brasilense, A. lipoferum and A. amazonense* (Tarrand et al., 1978; Hartmann, 1987; Reinhold et al., 1987). Nitrogenase activity is more sensitive towards salt stress than cellular growth on combined nitrogen (Rao and Venkateswarlu, 1985). Half-maximal in vitro nitrogenase activity of *A. amazonense* was obtained at 110 mM NaCl, as was the case with whole cell nitrogen fixation (Hartmann, 1987). In osmotic stress conditions, growth of *A. brasilense* and *A. halopraeferens* was stimulated by glutamate or proline, whereas no effect was found with *A. lipoferum* or *A. amazonense* (Hartmann, 1987). The latter two species eficiently use glutamate and proline as carbon and nitrogen sources. Therefore, *A. lipoferum* and *A. amazonense* might not be able to use amino acids as compatible solutes during osmotic stress. Betaine glycine (N, N, N - trimethyl glycine) stimulated nitrogen fixation in *A. brasilense* and *A. halopraeferens* at otherwise inhibitory osmotic stress. Even low concentrations of glycine betaine (0.1 mM) were sufficient to improve osmotolerance. *Azospirillum lipoferum* grew eficiently with choline and glycine betaine as sole source of carbon and nitrogen; accordingly, *A. lipoferum* could not use them as osmoprotectants. In contrast, *A. halopraeferens* had a high affinity uptake for choline (k_M: 16 µ*M*), and possibly oxidized it to glycine betaine, the most potent osmolyte (Imhoff, 1986). Obviously, osmotolerant strains scavenge potential osmo protectants such as proline, betaine or choline, originating from the plant. To take advantage of this relationship, the ability to synthesize betaines and proline by the plant partner should be better explored and improved cultivars used if possible.

Iron uptake properties

Iron is essential for aerobic life and also for biological biological nitrogen fixation. It is an essential component of nitrogenase and many enzymes involved in energy metabolism and reactions with oxygen. However, ferric

iron (Fe3+) is extremely insoluble in an aerobic environment; the solubility constant of ferric oxyhydroxy polymers is 10^{-38}. Therefore, most microorganisms produce and excrete low molecular weight molecules (for example, catechols, hy-droxamates), which can eficiently complex and solubilize iron (Neilands, 1984). These chelators (siderophores) are taken up by the microorganisms in speciic high affinity uptake systems, thereby assimilating the iron (Braun, 1985). In addition, roots of grasses excrete phytosiderophores, such as mugineic acid and avenic acid at iron-limiting conditions (Sugiura and Nomoto, 1984; Romheld and Marschner, 1986).

Different iron acquisition abilities in *Azospirillum*

The degree of establishment of a potentially beneficial microorganism, like *Azospirillum*, in the rhizosphere is expected to be influenced by its vigour for efficient iron supply in completition and or co-operation with other microorganisms and the plant. The potential for high affinity iron uptake of *Azospirillum* spp. was tested under severe iron limiting conditions, using high phosphate mineral medium (Albrecht and Okon, 1980) without any iron added and supple-mented with the artificial iron chelator 2, 2-dipyridyl. *Azospirillum* spp. was inhibited to various degrees. In *A. brasilense,* two groups with diferent iron assimila-tion efficiency existed. *A. brasilense* Sp245 and Nig5a grew reasonably well at iron stress conditions, whereas *A. brasilense* Sp7 and SpCd were relatively poor iron scavengers.

At iron stress conditions (closed symbols), $FeCl_3$ was omitted from the minimal medium (Albrecht and Okon, 1980) and the artificial iron chelator 2,2-dipyridyl (100 µ*M*) was added. Growth was performed in 20 ml cultures with shaking at 30°C (Au4: 41°C) and was measured turbidimetrically at 560 nm.

Utilization of microbial siderophores

The effect of foreign fungal and bacterial siderophores on growth of *Azospirillum* spp. was tested on iron-depleted minimal-medium plates containing 2, 2-dipyridyl. Micro-bial siderophores could usually not be used in this way with *A. lipoferum, A. halopraeferens* and *A. amazonense*. Again the two *A. brasilense* strains Sp7 and Sp245 showed a different response pattern. This again demon-strates the diferent ability of *Azospirillum* to face the iron problem. The utilization of ferrioxamine B, the main siderophore of *Streptomyces* spp., by some *A. brasilense* strains (for example, Sp245) may contribute to the successful colonization of a rhizosphere dominated by *Streptomyces*. Interestingly, the *A. brasilense* strain with superior iron acquisition properties (Sp245) is reported to become estabished in the rhizosphere more successfully as compared to the poor performer (Sp7) (Baldani et al., 1986). N_2-fixation occurs in many *Azospirillum* associations; but the basic question in its inoculation system remains as to how much nitrogen is contributed to the plant by the bacteria and under what growth condition, a factor that is highly variable and erratic.

Hormones

The ability to form plant hormones is a major property of many microorganisms and PGPB in general and specifically, species of *Azospirillum* that stimulate and facilitate plant growth (Tsavkelova et al., 2006). This is believed to be part of the mutualistic relationships developed between plants and their associate bacteria. *Azospirillum* spp. are known for their ability to produce plant hormones, as well as polyamines and amino acids in culture media (Hartmann and Zimmer, 1994; Thuler et al., 2003). *Azospirillum* pure culture produces mainly auxin like IAA (Lambrecht et al., 2000; Spaepen et al., 2007), other hormones detected at much lower, but biologically significant levels were Indole acetic acid (Tien et al., 1979), indole-3-butyric acid (IBA) (Fallik et al., 1989), indole-3-ethanol, indole-3-methanol (Crozier et al., 1988), unidentified indole compounds (Hartmann et al., 1983), several gibberellins (Tien et al., 1979; Bottini et al., 1989), abscisic acid (ABA) (Kolb and Martin 1985), and cytokinnins (Tien et al., 1979; Horemans et al., 1986).

Studies have suggested that the application of external hormones either synthetic or purified from bacterial culture, to seedlings completely reproduce the effects of *Azospirillum* on root developments and morphology (Zimmer and Bothe, 1988; Baca et al., 1994; Pattern and Glick, 1996). IAA is produced by the bacterium in vitro in large quantitites and is attributed to affect numerous alterations in plant functions yielding eventually growth function. IAA is produced during all stages of culture growth and well after the stationary phase (Malhotra and Srivastava, 2009). This feature makes the bacterium especially qualified for plant growth promotion when the effect last weeks or months after inoculation. Many studies have suggested the involvement of auxin produced by *Azospirillum* in root morphology (Baca et al., 1994).

Azospirillum has the capacity to synthesize and metabolize gibberillic acid (GA) in vitro (Bottini et al., 1989; Piccoli et al., 1996, 1997) and in planta (Bottini et al., 2004). A growth promotion effect of *Azospirillum* spp. on plants has been suggested to be partially caused by the production of GAs by the bacterium (Bottini et al., 2004). Involvement of GA A_3 (the main GA identified in *Azospirillum*) in promoting growth of maize was also suggested (Lucangeli and Bottini, 1997). However the study regarding the involvement of other phytohormones is still at an embryonic stage.

Azospirillum can produce NO at low oxygen pressure by denitrification (Hartmann and Zimmer, 1994). Nitric oxide (NO) is a volatile, lipophilic free radical which participates in metabolic, signaling, defense, and developmental pathways in plants (Lamattina and Polacco, 2007; Cohen et al., 2010). As its major role, NO participates in the IAA signaling pathways. This participation leads to lateral and adventitious root formation where the exact role of NO is as an intermediary in IAA-induced root development (Correa-Aragunde et al., 2006). All these data suggest a novel physiological role for NO in the organogenetic process leading to the establishment of root architecture in plants.

Mineral uptake enhancement

Azospirillum inoculation enhances mineral uptake in plants (Bashan and Levanony, 1990). *Azospirillum* inoculation resulted in uptake of N in wheat crops (Rai and Gaur, 1982). Both green house and field experiments showed that inoculation with *Azospirillum* results in enhance uptake of K^+, H_2PO_4 and other microelements (Fages, 1994). Inoculation may promote availability of ions in the soil by helping the plant scavenge limiting nutrients (Lin et al., 1983), which may explain the common accumulation of N compounds in the plant without any apparent N_2 fixation. Thus, the plant may absorb N more efficiently from the limited supply in the soil, resulting in a less N fertilization to attain a desired yield (Bashan and de-Bashan, 2010). But other workers showed that enhance growth of wheat and soya beans was not necessarily because of a general enhancement of mineral uptake (Bashan et al., 1990). This controversial phenomenon has modestly been pursued in recent years.

Plant disease control

Azospirillum is not yet considered to be a classical biocontrol agent of soil borne plant pathogen as many strains lack direct suppressive chemicals or hydrolytic enzymes likely to affect plant pathogens. However, there have been reports on moderate capabilities on *A. brasilense* in biocontrolling crown gall producing agrobacteria (Bakanchikova et al., 1993); bacterial leaf blight of mulberry (Sudhakar et al., 2000) bacterial leaf and/or vascular tomato diseases (Bashan and de-Bashan, 2002a) and *Pseudomonas syringae* pv. tomato, causing bacterial speck of tomato (Bashan and de-Bashan, 2002b).

The effect of *Azospirillum brasilense on* crown gall formation in dicotyledonous plants was studied after inoculating them with virulent strains of *Agrobacterium tumefaciens.* When the wounded tissues of grapevines and carrot disks were preinoculated with the live cells of *A. brasilense* 94-3 or Sp-7, the development of the typical bacterial galls was inhibited and the protective effect of *Azospirillum* lasted over a 24-hrs period (Bakanchikova et al., 1993). Inoculation of *A. brasilense* sp 245 on micropropagated plants of *Prunus cerasifera* L. clone Mr S 2/5 protected the plant from various fungal rhizospheric community which has been confirmed by denaturing gradient gel electrophoresis (DGGE) profile of the rhizospheric microbial community (Russo et al., 2008). The antibacterial activities exhibited by *Azospirillum* have been found to be related with its ability to synthesize bacteriocins (Tapia-Hernández et al., 1990), siderophores (Tapia- Hernandez et al., 1990; Perrig et al., 2007) and phenylacetic acid (PAA), an auxin-like molecule with antimicrobial activity (Somers et al., 2005). These and other associated study thus suggests that species of *Azospirillum* as inoculants could be used in the management of various plant diseases.

Productions of vitamins and toxic residue degradation

Productions of vitamins by *A. brasilense* and their liberation viz, thiamine, niacin, pantothenic acid and riboflavin in large quantities (Rodelas et al., 1993) as was riboflavin (Dahm et al., 1993) were significantly affected by the presence of different carbon sources and the age of the culture. *A. lipoferum* can reduce 4-chloro-nitro-benzene, an aromatic compound used in the manufacturing of pesticides, dyes, explosives and industrial solvents which is an environmental pollutant (Russel and Muszynski, 1995). Cotton plants could be partly protected from harmful effects of the herbicide 2, 4-D by inoculation with *A. brasilense*. The degrading plasmid of 2, 4-D was transferred into *A. brasilense* Sp7. Trans-conjugants degraded 2, 4-D in pure culture via cometabolism. However, when the transconjugants were inoculated on cotton seeds, the plants were resistant only to low levels of the herbicide, which is not sufficient for protection of cotton. Plants growing in soils with this concentration of herbicide and inoculated with wild-type strains died (Feng and Kennedy, 1997).

Synthetic inoculants

Since the introduction of new synthetic inoculants of *Azospirillum* made up of alginates (Bashan, 1986) it was being commercially applied. These crop inoculants were developed with an *A. lipoferum* strain. The process involves the entrapment of living cells in alginates beads and air dehydration. Air dehydration unfortunately eliminated the vast majority of the original cells, but the remaining cells were sufficient to serve as an inoculant (Paul et al., 1993). Unfortunately, to the best of our knowledge these French registered inoculants were

never released in to the market (Fages 1994). In 1997 in Belgium the commercial inocula AZOGREEN-m (Liphatech, France), which is a peat-based inocula containing the strain *A. lipoferum* CRTI, was used in a field experiment on grain maize *Zea mays* cv. Kajak (Dobbelaere, 2001). Different levels of N fertilizer were also applied there. The aim was to evaluate the performance of these commercial inoculums on maize. The final concentration of bacteria added was 10^6 CFU per seed, which might be too low to obtain plant growth promotion. On average, the grain yield and N content in the grains were higher for the inoculated plants, but the differences were not significant. However, there was a clear effect on the uptake of N by inoculation.

Para-nodulation in non-legumes

A promising alternative approach to solve the problem of low frequency nodules on non-legumes is to artificially induce nodule like structures and stimulate the diazotrophs to colonize these structures (as protective niche). The term *para*-nodules was introduced by Tchan and Kennedy to describe the chemically induced nodules, since they differ from the naturally occurring legume nodule (Kennedy and Tchan, 1992). These induced nodule-like outgrowths are modified lateral roots with carbon reserves (as starch in amyloplasts) similar to those found in the cortex of roots, and microorganisms are able to modulate or interfere with the development of these outgrowths (Ridge et al., 1992). Hence induction of nodules like structures or *para*-nodules on cereal roots is gaining momentum. Formation of nodular structures on roots of non-legumes and cereals has been reported since long, but without any inference, that these may contain bacteria. Induction of *para*-nodules by 2-4-D treatment and its colonization by microbes was recognized, but without any acceptable evidence of N_2-fixation (Nie et al., 1992). These *para*-nodules when colonized by the diazotrophic bacteria *Azospirillum*, fix atmospheric nitrogen within these *para*-nodules (Kennedy and Islam, 2001). The inoculated bacteria enter through loosening of the epidermis without causing much damage to infected host epidermal cells (Christiansen-Weniger and Vaderleyden, 1994; Elanchezhian and Panwar, 1997). The bacteria colonize intercellularly usually in the basal zones of the *para*-nodules (Kennedy et al., 1997). It was found that non-legumes treated with 2,4-D alone didn't show any improvement over control until the presence of *Azospirillum* (Panwar and Elanchezhian, 1998). However, it was found that *para*-nodules colonized by the bacteria intracellularly promote a higher level of nitrogen fixation; nitrogen becomes easily available which results in higher yield (Saikia et al., 2004). Host plants benefit from enhanced nitrogen fixation in their roots with *para*-nodules because fixed nitrogen is incorporated into the host plant. *Para*-nodules can add a new dimension to research on biological nitrogen fixation, but extensive developmental and biochemical modification of the *para*-nodule system is required before effective nitrogen fixation can be achieved. The options are intriguing (Kennedy and Tchan, 1992; Christiansen-Weniger, 1994; Kennedy, 1994).

Effect of *Azospirillum* inoculation on non legumes

Azospirillum was initially tested for agronomic exploitation more than a decade ago as a result of two basic features: (i) its ability to fix atmospheric N and, (ii) its intimate association with roots of cereals and grasses. Although no special morphological structure was ever found in or on inoculated roots, increase in N content of inoculated plants as well as reported yield increase prompted numerous field inoculation attempts.

Since the 1980's, the response of agriculturally important crops to inoculation with *Azospirillum* was investigated in numerous field and greenhouse experiments carried out in various countries. The results from these field experiments were evaluated in several reviews (Bashan and Levanony, 1990; Okon and Labandera-Gonzalez, 1994; Dobbelaere and Okon, 2003). Based on published data, it was concluded that inoculation with *Azospirillum* resulted in significant yield increases in the magnitude of 5 to 30% in about 60 to 70% of the experiments, with often even greater increase under greenhouse conditions (Sumner, 1990; Okon and Labandera-Gonzalez, 1994). Inoculation experiments were carried out under controlled and field conditions, using different plant-growth systems and plant species.

Azospirillum inoculation on non-leguminous plants results in significant changes in various plant growth parameters, which however may or may not affect a crop yield. Earlier workers reported the following above ground plant growth responses to *Azospirillum* inoculation in non-legumes: increase in total plant dry weight, in the amount of nitrogen in shoots and grains, and in the total number of tillers, fertile tillers and ears, earlier heading and flowering time, increased number of spikes and grains per spike, increased grain weight, greater plant height and leaf size, and higher germination rates (Baldani and Döbereiner, 1980; Kapulnik et al., 1981; Mertens and Hess, 1984; Millet and Feldman, 1986; Warembourg et al., 1987).

In recent years *Azospirillum* sp. co-inoculation with other symbiotic microorganisms creates a successful system of BNF in non legumes which can lead to many profits for plants. Co-inoculation of wheat seeds with *A. brasilense* and *R. meliloti* had positive and significant effects on the grain yield and N, P and K content of the wheat grains in compared to either single inoculation or control plants. This was due to increase in number of grains (26%) and grain weight per plant (22%) in co

inoculated plants. And it was also found that co inoculation could result in higher grain yield with better quality in comparison to single inoculation (Askary et al., 2009). Inoculation of AM fungi and *Azospirillum* on seedlings of Onion (*Allium cepa* L.) played a vital role in supplying N and P to the onion and found enhancing the growth and yield over the untreated control (Sridevi and Ramakrishnan, 2010). Under green house condition also co inoculation of *Azospirillum* sp. with methyloptrophic bacteria *Methylobacterium oryzae* in tomato, red pepper and rice increased plant growth viz, shoot or root length and increased the nutrient uptake compared to single inoculated or uninoculated plants. Also co inoculation increased the MPN concentration, the activity of nitrogenase, urease and phosphatase enzyme in soil when compared to uninoculated control or individual inoculation (Madhaiyan et al., 2009).

Effects of *Azospirillum* inoculation on root morphology

Root hairs

One of the most pronounced effects of inoculation with *Azospirillum* on root morphology is the proliferation of root hairs. Inoculation of several cultivars of wheat, maize, tomato, sorghum, foxtail millet, pearl millet and other grasses with several strains of *Azospirillum* caused morphological changes in the root, including an increase in the number and density of root hairs and a shortening of the time of appearance of root hairs (Tien et al., 1979; Okon, 1984; Kapulnik et al., 1985b; Hadas and Okon, 1987; Morgenstern and Okon, 1987a; Barbieri et al., 1991). Inoculation further increased the length of mature root hairs and shortened the distance between the root apex and the region at which root hairs start to elongate (Harari et al., 1988; Dobbelaere et al., 1999). This effect on root hair proliferation was strongly influenced by, and varied with, inoculum level (Kapulnik et al., 1985a; Okon and Kapulnik, 1986; Hadas and Okon, 1987; Dobbelaere et al., 1999). Most of this stimulation of root growth is assumed to result from the production of phytohormones by the bacterium, as the morphological changes of the plant root following *Azospirillum* inoculation could be mimicked by applying a combination of plant growth substances (Jain and Patriquin, 1985; Tien et al., 1979). Promotion of root growth, especially increase of hair density in zones physiologically active for nutrient uptake and water absorption, could lead to better soil exploration, and improve the growth and development of the plants (Fulchieri et al., 1993).

Root hair deformation and branching

Apart from promoting root hair development, inoculation with *Azospirillum* (10^9 – 10^{10} cfu ml^{-1}) also caused branching of root hairs. Two types of root hair branching was distinguished in wheat; branches of equal length (the tuning-fork like deformation) and branches of unequal length (Patriquin et al., 1983). This root hair deformation was found to be somehow strain-specific, with homologous strains (isolated from surface-sterilized roots of the same crop as that to be inoculated) inducing the formation of more tuning forks than non-homologous strains. No or very few tuning forks were found in non-inoculated plants (Jain and Patriquin, 1984). The role, if any, of root hair branching in the colonization of roots by *Azospirillum* is not known. The phenomenon is significant, however, in that it is a predictor of the potential growth response of wheat to inoculation with *Azospirillum* (Jain and Patriquin, 1984). Strains that caused the most tuning forks in the laboratory also brought about the greatest increase in plant N in a separate field experiment (Baldani et al., 1983).

Cross sections

Cross-sections of corn and wheat roots inoculated with *Azospirillum* showed an irregular arrangement of cells in the outer four or five layers of the cortex (Lin et al., 1983; Kapulnik et al., 1985b). Cross sections taken near the root tip shortly after inoculation of burr medic with 10^9 cfu ml^{-1} of *Azospirillum brasilense* strain Cd, showed larger cortical cells. However, their number (in cross sections) did not increase, as compared to controls (Yahalom et al., 1991). Similarly, DNA concentration in root segments of burr medic inoculated with 10^9 cfu ml^{-1} of *Azospirillum* was significantly lower than in roots inoculated with 10^7 cfu ml^{-1} of *Azospirillum* or than in controls. From this finding it was concluded that the reduction in root growth might be the result of a decreased cell division in the apical meristem of the root (Yahalom et al., 1991). These effects of inoculation on cell arrangement and size may be due to the production of plant-growth promoting substances by the colonizing bacteria, or by the action of pectic enzymes produced by the bacteria (Okon and Kapulnik, 1986).

Root elongation

Besides stimulating root hair formation, inoculation with diazotrophs can also promote the elongation of primary roots. Increases in root length were observed after inoculation of several cultivars of wheat, sorghum, proso millet, sugar beet, rice and tomato with *A. brasilense* (Kapulnik et al., 1985a; Hadas and Okon, 1987; Murty and Ladha, 1988; Levanony and Bashan, 1989; Sarig et al., 1992). Here again, the effect is dependent on the bacterial concentration that is applied. Low concentrations (10^3 – 10^6 cfu ml^{-1}) stimulated root elongation and high inoculum concentrations (above 10^7

cfu ml^{-1}) inhibited root growth (Kapulnik et al., 1985b; Harari et al., 1988). The concentration dependent effect on root length is consistent with the effects of plant root treatment with high concentrations of exogenous IAA. Depending on the plant species, exogenous IAA concentrations above a threshold of about 10^{-6} to 10^{-9} M are inversely proportional to root elongation (Pilet and Saugy, 1985). By using a mutant strain of *A. brasilense* impaired in IAA production, it was indeed demonstrated that the effect on wheat root elongation upon inoculation was mainly due to the production of IAA by this bacterium (Dobbelaere et al., 1999).

Root Branching/lateral root formation/adventitious root formation

Inoculation of wheat seedlings with *A. brasilense* produced an increase in the number and length of the lateral roots as a plant response (Barbieri et al., 1986, 1988). Similar results were obtained on pearl millet by Tien et al. (1979), on hybrid *Sorghum bicolor x Sorghum sudanense* by Morgenstern and Okon (1987a) and on sugar beet by Kolb and Martin (1985) and Marschner et al. (1986). Yahalom et al. (1991) found that inoculation of burr medic seedlings grown in pouches with *A. brasilense* strain Cd at a concentration of 10^6 cfu ml^{-1} significantly increased the number of lateral roots.

In a field experiment with wheat, inoculation with *Azospirillum* was found to increase the total number of roots per plant as well as the number of roots per tiller (Kapulnik et al., 1987). In a maize field experiment with *A. brasilense* strain Cd carried out in Israel in 1998, it was observed that the number of adventitious roots and the total adventitious root length increased significantly above non-inoculated controls by 24 and 41%, respectively, 2 weeks after emergence (Dobbelaere et al., 2001).

Effect on root function

Respiration and Respiratory Enzymes

In a Petri dish system, inoculation of tomato with 10^8 cfu ml^{-1} of *A. brasilense* strain Cd increased the total respiration rate per root by 70% over non-inoculated controls (Hadas and Okon, 1987). Respiration rates of sorghum (Sarig et al., 1992), maize and common bean (Vedder-Weiss et al., 1999) roots were also increased upon inoculation with *Azospirillum*. An increased root respiration rate indicates an increase in metabolic activity. The specific respiration rate, expressed as micromoles of O_2 per minute and per milligram of root dry weight, was significantly lower in inoculated roots, suggesting that less energy was spent per gram of dry material that was formed.

Application of *A. brasilense* to maize plants at an inoculum concentration of 10^7 cfu $plant^{-1}$ led to an increase in the specific activity of several enzymes in root extracts, including alcohol dehydrogenase, glutamine synthetase, isocitrate dehydrogenase, malate dehydrogenase, pyruvate kinase and shikimate dehydrogenase. An increase in the specific activity of these enzymes was observed in inoculated roots between the 2nd and 3rd week after sowing as compared to non-inoculated controls (Fallik et al., 1988).

Phosphatase

Acid phosphatase is involved in the breakdown of organic phosphate compounds. The specific activity of acid phosphatase, in extracts of inoculated maize roots, significantly increased at the 2nd, 3rd and 4th week after sowing as compared to that in non inoculated controls (Fallik et al., 1988). Thus, the increased phosphate uptake observed in roots inoculated with *Azospirillum* could result from an increase in acid phosphatase activity

Auxin, gibberellin and ethylene plant metabolism

A. brasilense was found to affect the amount of free and bound IAA in the inoculated roots of maize seedlings (Fallik et al., 1989). Normally about 95-98% of the IAA in maize seed is found as an ester conjugate of IAA (Epstein et al., 1980), which is then metabolized during seed germination to give active free IAA (Nowacki and Bandurski, 1980; Nonhebel et al., 1985). Inoculation of maize seedlings with *Azospirillum* resulted in a simultaneous decrease in the amount of conjugated IAA and significant increase in the free IAA concentration compared to non inoculated control roots. When IAA is supplied to non-inoculated plant roots it is usually conjugated rapidly (Bandurski, 1980). The presence of free IAA in the inoculated roots, which were fed, with IAA for 24 h and its absence from the non-inoculated control may be attributed to hydrolysis of the conjugated IAA by the bacteria to release free IAA, or to blocking of conjugation of the exogenous IAA by the bacteria.

Inoculation with *A. lipoferum* had substantial effects on the gibberellin (GA) content of the roots of corn seedlings (Fulchieri et al., 1993). GA_3 was found as the free acid in extracts of roots of seedlings inoculated with *A. lipoferum*, while in extracts of roots of non-inoculated plants it was only found after hydrolysis of the glucosyl conjugate fraction.

In all these cases it is not clear if the relative higher amounts of free IAA and GA_3 in the root tissue of inoculated maize were derived i) from plant growth promoting substances (PGS) excreted by the colonizing bacteria, ii) by changes in plant hormone metabolism caused by excretion of PGS by the bacteria, iii) by higher

respiration rates of the roots demanding more glycosidic residues from hydrolysed hormonal conjugates, thus freeing IAA and GA_3 or iv) by enzymes liberated by the bacteria that are responsible for de-conjugation of glucosylated forms. Hydrolysis of gibberellin-glucosyl conjugates by *A. lipoferum* has been reported by Piccoli et al. (1997), suggesting that the growth promotion in plants that is induced by *Azospirillum* infection may occur by a combination of both gibberellin production and deconjugation of plant-derived gibberellin-glucoside/ glucosyl ester by the bacterium.

Exposure to *Azospirillum* at a concentration of 10^9 cfu ml^{-1} (in the absence of *Rhizobium*) or to compounds excreted by the bacteria into the growth medium caused a 40% increase in endogenous ethylene production by the roots of burr medic (Yahalom et al., 1990). A less concentrated inoculum did not increase ethylene production. As IAA can stimulate ethylene production, it was suggested that probably the high exogenous IAA level caused an increase in ethylene level in the plant.

Mineral uptake

Enhanced mineral uptake in plants inoculated with *Azospirillum* and other diazotrophs has been reported repeatedly, both in greenhouse experiments (Lin et al., 1983; Kapulnik et al., 1985a; Morgenstern and Okon, 1987b) and in the field (Sarig et al., 1984; 1988; Kapulnik et al., 1987; Fages, 1994). The major nutrient involved was nitrogen in the form of nitrate in wheat, sorghum, corn and *S. bicolor x S. sudanense* plants (Boddey and Döbereiner, 1988; Kucey, 1988) or ammonium in rice plants (Murty and Ladha, 1988). Also improved uptake of $H_2PO_4^-$ (Lin et al., 1983; Murty and Ladha, 1988; Sarig et al., 1988), K^+ (Lin et al., 1983), Rb^+ (Morgenstern and Okon, 1987b), and Fe^{2+} (Barton et al., 1986) by inoculated plants has been demonstrated. However, it is not yet generally accepted that this improved nutrient uptake is due to a specific enhancement of the normal ion uptake mechanism. On one hand it was suggested that increased mineral uptake by inoculated plants is the consequence of a general increase in the volume and surface of the root system, as reflected by increased root hair formation and an increased root number, thickness and length (Gunarto et al., 1999; Biswas et al., 2000). Higher K^+ and Fe^{2+} uptake for instance are related to thicker roots (Barber, 1985) and higher $H_2PO_4^-$ uptake to the presence of root hairs (Gahoonia and Nielsen, 1998; Gahoonia et al., 2001). Concomitant increases in root elongation; root surface area and mineral uptake were indeed reported after inoculation of wheat with a mixture of *A. brasilense* strains (Kapulnik et al., 1985a, b). Using a hydroponic system containing NO_3^-, both the surface area of wheat roots and the uptake of NO_3^- from the mineral solution during plant growth were found to increase upon inoculation. However, no significant changes were obtained in the NO_3^- uptake root surface area ratio, indicating that the increased NO_3^- ion uptake by wheat inoculated with *Azospirillum* was due to general increase in root surface area, and not because of an increase in the specific uptake rate (Kapulnik et al., 1985a). Murty and Ladha (1988) found enhanced NH_4^+ and $H_2PO_4^-$ ion uptake by rice plants inoculated with *Azospirillum* without concomitant increase in the root surface area and Lin et al. (1983) demonstrated that *Azospirillum*-inoculated corn and sorghum plants took up minerals (N, P and K) from solutions at faster rates than uninoculated controls.

With respect to this, it has been reported that inoculation with *A. brasilense* Cd strain resulted in a reduction in the membrane potential of the root cells of soybean seedlings and a significant increase in the proton efflux of the roots of wheat seedlings (Bashan et al., 1989; Bashan, 1991). These activities were proposed to be responsible for the increase in mineral uptake by *Azospirillum*-inoculated plants. Proton extrusion through membranes of root cells, which results in acidification of the rhizosphere, is supposed to be a major mechanism in the mobilization of minerals in plants (Spanswick, 1981; Marschner et al., 1986). In hydroponic systems, the enhanced specific uptake of minerals from the solution was concomitant with improved root hydraulic conductivity (25-40% increase as compared to controls) observed in sorghum roots (Sarig et al., 1992). Alternatively, the surface activity involved in ion uptake may have increased as a result of the altered cell arrangements in the outer four or five layers of the root cortex, as was observed in cross sections of inoculated plants (Lin et al., 1983; Kapulnik et al., 1985b; Yahalom et al., 1991). These morphological changes were found to have a physiological effect on inoculated roots: the altered area stained darker with methylene blue, suggesting an increase in the surface activity involved in ion uptake (Lin et al., 1983).

Recently, a gene encoding an ammonium transporter in the root hairs of tomato (*LEAMTI;2*) has been identified. Inoculation of N-depleted tomato plants with *A. brasilense* or *Azoarcus* sp. induced *LEAMT1; 2* expression, while *A. brasilense nif*DK mutants failed to do so (Becker et al., 2002). This indicates that NH_4^+, resulting from nitrogen fixation by the bacteria, might be used as an N-source under these conditions. On the other hand, the transported NH_4^+ ion itself could represent a key signal in the associative interaction between higher plants and N_2-fixing microorganisms. Induction of ammonium transport in root hairs can lead to enhanced nutrient uptake by tomato roots and consequently promotion of plant growth upon inoculation.

Water uptake

The larger root system of inoculated plants does not only

lead to enhanced mineral uptake, water uptake can also be improved, which in turn could benefit growing of crops in water-deficient soils. In field experiments the water regime of sorghum plants was improved by inoculation, as seen in their higher leaf water potential, lower canopy temperatures and greater stomatal conductance and transpiration. Total extraction of soil moisture by inoculated plants was greater (by about 15 %) and occurred from deeper soil layers as compared with non-inoculated controls. These findings indicate that inoculation with *Azospirillum* could lead to yield increases in dry land grain sorghum, primarily through improved utilization of soil moisture (Sarig et al., 1988; Fallik et al., 1994).

Inoculation with *A. brasilense* also delayed leaf senescence in sorghum plants subjected to osmotic stress, indicating an improved water uptake in the inoculated stressed roots (Sarig et al., 1990).

Leaf area

Inoculation of sorghum with *A. brasilense* grown in hydroponic systems significantly enhanced leaf area development mainly at 24 to 28 days after emergence (Sarig et al., 1990). At later stages, leaf senescence was delayed in inoculated plants, thus favouring dry matter accumulation and grain filling.

Mineral content

As mentioned earlier, inoculation with *Azospirillum* and other diazotrophs can result in an increase in the mineral uptake by plants. Whether this is due to the specific enhancement of the normal ion uptake mechanism or due to the promotion of root development, the net result is an increase in the mineral content of inoculated plants. It not only increases N but also the content of other elements like P and K was found to have increased (Sarig et al., 1984; Yahalom et al., 1984; Kapulnik et al., 1987; Boddey et al., 1986; Boddey and Döbereiner, 1988; Sumner, 1990). Inoculation of a local maize variety in India with strains of *A. chroococcum* or *A. brasilense* resulted in significantly higher values for N and P content of plant components (Pandey et al., 1998).

However, the incidence of positive results may not be frequent enough to enable commercialization of the bacterial population.

Conclusion

Available literature on the role of *Azospirillum* in non-legumes reveals that Azospirilla definitely plays an active role in the growth and development of non legumes. However there are many challenges to be faced in maximizing and popularizing BNF using *Azospirillum.* *Azospirillum* being the most studied plant associative bacteria, its prospects of using for the benefit of mankind are greater than those of other bacteria but not in the immediate future. Further modification of this bacterium will be a useful strategy to meet the continuous increase of nitrogen demand in the world. An expanding knowledge base and the use of powerful tools of biotechnology may help in gaining further insight into the application of *Azospirillum* as inoculants. These include sequencing and evaluation of entire genomes of plants such as rice, sugar cane, maize and others, as well as the use of modern techniques for high-throughput analysis (microarray) for transcriptomics and proteomics, will detect changes in inoculated plants (PGPR-plant associations) faster and more accurately, yielding a vast information on the interactions.

Therefore efforts should be taken regarding certain issues such as:

i. As fertilizer N generally inhibits BNF in both symbiotic and non symbiotic systems, efforts should be made to reduce those effects of N application.
ii. Most *Azospirillum* strains can fix nitrogen but only a fraction of it, if any at all is transferred to the plant. So efforts should be made to pin point factors causing this low nitrogen fixation activity in non-legumes.
iii. Development of cultivar-strain resistant to stress conditions, acid and alkaline soils, nutritional deficiency, salinity, high temperature, presence of toxic elements etc.
iv. To get maximum benefit out of *para*nodule formation and its colonization by *Azospirillum* by the development of different biochemical approaches and extension of this technique to farmer's field.
v. Extensive research should be done on the morphology and physiology of plants with more emphasis on root architecture, root function, biological control, and plant hormone metabolism.

Moreover, extensive field experiments, are also needed which will allow the discovery of new strains of Azospirilla and also the discovery of Azospirilla-genotype combinations that shows little or no N-deficiency symptoms in the absence of N fertilizer. Testing of mixtures of these strains for effects on yield as well as testing the inoculums dose required for better yield responses, also need to be studied properly and elaborately. Plant breeding for increased N_2 fixation and comparison of such genotypes with non-fixing genotypes will help us to identify the most efficient plant genotype-*Azospirillum* combination. It is suggested that the direction in which *Azospirillum* research should proceed, to gain the full potential of this association, is towards more basic understanding of the underlying fundamental components of the system and less towards full-scale field experiments. We assume that this approach will be the best in ultimately harnessing *Azospirillum* activity for the benefit of mankind.

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