

Samina Mehnaz

Abstract

Azospirillum is known for its nitrogen-fixing and phytohormone production ability. It is one of very well-studied plant growth-promoting rhizobacteria, at lab scale to field. None of its species or strain is reported as human or plant pathogen. It is considered as safest bacteria which can be used as a biofertilizer at commercial level for several crops, especially cereals or grasses including wheat and rice which are of economic importance for the whole world. Some of its species are reported for phosphate-solubilizing ability and high salt tolerance. Fifteen of its species have been isolated from variety of hosts and environmental sources; however, a majority have been reported from plants. There are several reviews available on this organism; in this chapter, an overview of this organism covering its plant growth-promoting abilities, used as inoculum in lab and field experiments and used as a commercial biofertilizer for different crops, is provided.

Characteristic Features of Genus *Azospirillum* and Its Species

Azospirillum is a gram-negative curve rod, micro-aerophilic, non-fermentative, and a chemoorganotroph that belongs to class *Alphaproteobacteria*. This bacterium was first time isolated, reported, and named in 1978 (Tarrand et al. 1978). As the name reflects, it was described as nitrogen-fixing, curved

rod-shaped organism. Two species, *A. lipoferum* and *A. brasilense*, were designated at that time. Since then, 15 species of this genus have been described. For a long time, only five species were known, but during the last 10–12 years, ten new species have been described. *Azospirillum* is known to be an organism which has been isolated from most of the parts of the world, mainly from rhizosphere and roots of the members of the family Gramineae including rice, wheat, corn, and sugarcane as most common hosts. Coffee, fruits, and orchids have also been reported as host of this bacterium but less frequently as compared to grasses. Two new species *A. picis* and *A. thiophilum* had been isolated from very different environment, i.e.,

S. Mehnaz (✉)
Department of Biological Sciences, Forman Christian College (A Chartered University),
Ferozpur Road, Lahore 54600, Pakistan
e-mail: saminamehnaz@fccollege.edu.pk;
saminamehnaz@gmail.com

road tar and sulfide spring (Lin et al. 2009; Lavrinenko et al. 2010).

Azospirillum was first time described as a nitrogen fixer – ability to convert atmospheric nitrogen into ammonia. Later on, the second most common characteristic feature was phytohormone production, specifically auxins. Among 15 species, *A. palatum* is an exception, lacking the ability to fix nitrogen, as reduction of acetylene (method for the detection of nitrogen fixation) was not detected by it (Zhou et al. 2009). The production of indoles is not reported for *A. picis* and *A. palatum*. The difference in carbon source utilization is helpful for distinguishing among species. Most common carbon sources are sugars and sugar alcohols. Reis et al. (2011) provided a very comprehensive detail on their carbon source utilization.

During the last 3 years, genomes of *A. lipoferum* 4B (www.genoscope.cns.fr), *A. brasilense* CBG497, *A. brasilense* Sp245, *A. amazonense* Y2, and *Azospirillum* sp. B510 have been sequenced and published (Kaneko et al. 2010; Sant'Anna et al. 2011; Wisniewski-Dye et al. 2012). These sequences provided the insight into genetics of this genus. This information can be used not only to improve the strains but also to explore their potential for purposes other than agriculture, e.g., bioremediation. A comprehensive review about genetic and biochemical traits of *Azospirillum* has been published by Steenhoudt and Vanderleyden (2000). Recently, Reis et al. (2011) discussed about the genetic makeup of *Azospirillum*, mostly based on genome of *Azospirillum* sp. B510. In addition to *nif* gene cluster, *nod* gene has also been identified in *Azospirillum* spp. Genes for indole acetic acid (IAA) production and those involved in chemotaxis signal transduction are also reported. According to information based on *Azospirillum* sp. B510 genome, this genus has nine sets of *rrns* genes which is atypical for the members of α -proteobacteria. Quorum sensing is a very rare feature for this genus. Boyer et al. (2008) reported the N-acyl-L-homoserine lactone (AHL) production in two strains of *A. lipoferum*.

If we look at the published material about this genus, most of the information is based on two species, i.e., *A. brasilense* and *A. lipoferum*. It

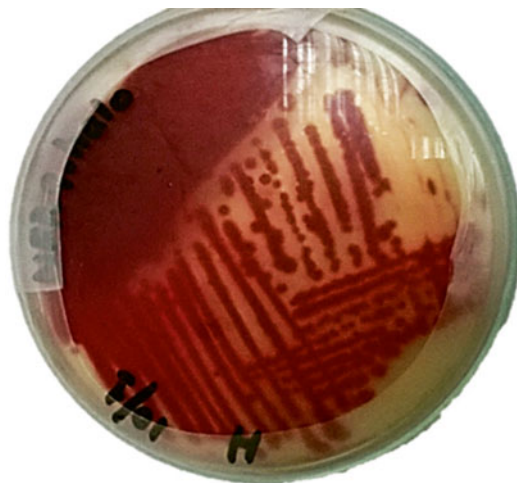


Fig. 15.1 Pure culture of *Azospirillum halopraeferens* NFP9 on LB medium (Mehnaz et al. unpublished results)



Fig. 15.2 Single cell of *Azospirillum zeae* N7 (Mehnaz et al. 2007a)

can be due to worldwide occurrence of these two as compared to the other species. *A. irakense* has not been reported from anywhere after its first isolation. Reports for *A. amazonense* and *A. halopraeferans* are also rare (recently authors' lab has isolated a strain of *A. halopraeferans* from *Kochia indica*; Fig. 15.1, unpublished results). Two of the species, *A. canadense* and *A. zeae*, have been isolated and designated by our group (Mehnaz et al. 2007a, b; Fig. 15.2) and not reported yet from anywhere else. Similarly, the rest of the species have been recently designated;

therefore, commenting on their occurrence is too early to discuss.

Growth-Promoting Abilities of *Azospirillum*

Among plant growth-promoting rhizobacteria (PGPR), *Azospirillum* is one of those bacteria which had never been reported as a pathogen for any living organism. It has been used as an inoculum for several crops due to its beneficial effects. These beneficial effects can be an individual or a combined effect of several mechanisms which exist in this bacterium. *Azospirillum* is known for nitrogen fixation, phytohormone production, phosphate solubilization, and siderophore production. It was never considered as a biocontrol agent although there were always reports available about its anti-pathogenic activity. ACC deaminase activity has not been reported for this genus, and quorum sensing is very rare as mentioned earlier. The growth-promoting attributes of *Azospirillum* are described below in detail.

Nitrogen Fixation

The most important characteristic feature of *Azospirillum* is its ability of biological nitrogen fixation (BNF). Nitrogen fixation is common among bacteria responsible for symbiotic relationship with leguminous plants. These bacteria are known for their endophytic nature and nodule formation and inability to fix nitrogen “in vitro.” In contrast, *Azospirillum* is known as a “free-living” nitrogen fixer that does not make nodules. It stays in close association with the plant, in the rhizosphere, and on the root surface.

The most common method to detect the nitrogen fixation in bacteria is acetylene reduction assay. Nitrogenase enzyme responsible for nitrogen fixation has also the ability to convert the ethylene into acetylene. Acetylene reduction assay is based on it. N¹⁵ dilution method has also been used by several researchers and considered very authentic when it comes to quantifying the contribution of these microorganisms to the plants in

terms of nitrogen and minimizing the use of N fertilizers. There is no doubt about the nitrogen-fixing ability of *Azospirillum*, but how much of the fixed nitrogen is transferred to the plants is still a big question. Transfer process is very slow, and a very small part becomes available to the plant. The death and subsequent mineralization of dead nitrogen fixers can release significant amount of fixed nitrogen (Okumura et al. 2013).

Phytohormone Production

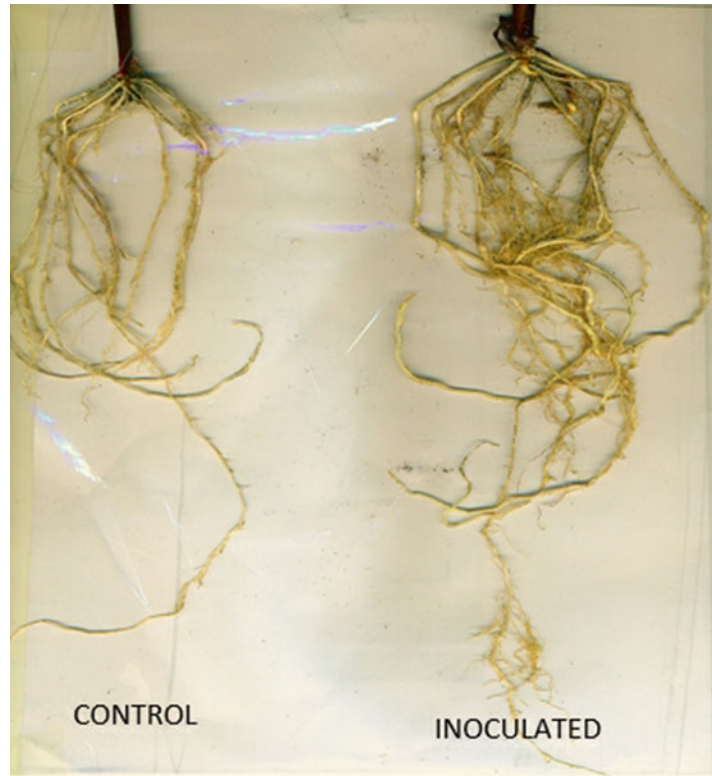
Almost 80 % of soil bacteria have the ability to produce phytohormones, and *Azospirillum* is one of them. Phytohormones are divided into five classes, i.e., auxins, gibberellins, cytokinins, abscisic acid, and ethylene. *Azospirillum* is known for the production of all of these except ethylene.

Auxins

Auxins are responsible for division, extension, and differentiation of plant cells and tissues. These compounds increase the rate of xylem and root formation; control vegetative growth, tropism, and florescence; and affect photosynthesis, pigment formation, biosynthesis of several metabolites, and resistance to biotic stress (Bashan and de-Bashan 2010). Among auxins, IAA production is very high in this genus, and it follows more than one pathway.

IAA production by using tryptophan is known since 1979, for *Azospirillum* (Reynders and Vlassak 1979). IAA production depends on composition of medium, pH, and availability of tryptophan as precursor (Ona et al. 2003; Malhotra and Srivastava 2006, 2008). Spaepen et al. (2007) characterized IAA production via indole-3-acetamide (IAM) and indole-3-pyruvate (IPyA) intermediates, in *Azospirillum*. Very low level of pyridoxine and nicotinic acid increased IAA production in *A. brasilense*, indicating that vitamins may also regulate IAA synthesis (Zakharova et al. 2000). IAA is produced during all growth stages of *Azospirillum* and most after the stationary phase (Malhotra and Srivastava 2009). Due to this feature, credit of growth promotion by

Fig. 15.3 Effect of *Azospirillum* sp. inoculation on root growth of corn plants (Mehnaz et al. unpublished results)



Azospirillum inoculation is given to IAA as effect lasts longer. Several auxins including indole butyric acid, indole lactic acid, indole acetamide, indole acetaldehyde, indole ethanol, indole methanol, and phenyl acetic acid (PAA) have been detected in *Azospirillum* cultures (Hartmann et al. 1983; Crozier et al. 1988; Fallik et al. 1989; Costacurta et al. 1994; Somers et al. 2005).

Plants inoculated with *Azospirillum* strains showed increase in number and length of root hairs, number of lateral roots, diameter and length of lateral and adventitious roots, and root surface area. IAA produced by *Azospirillum* is considered as the major mechanism responsible for increase in root system (Fig. 15.3; Mehnaz et al., unpublished results). Another possibility is that inoculation enhances the endogenous level of IAA in plant. The best way to prove the direct involvement of IAA produced by *Azospirillum* on plant growth is to develop IAA-deficient mutant and use them as inoculant, but due to existence of more than one pathway, it is almost impossible to make a mutant which does

not produce IAA (Zakharova et al. 1999; Spaepen et al. 2007).

Gibberellic Acids (GAs)

GAs are responsible for cell division, cell elongation, and effects on different growth stages of plants. GAs are involved in breaking dormancy during seed germination. These compounds induce synthesis of α -amylase which hydrolyzes starch into glucose. *A. brasilense* is known to enhance germination of soybean and wheat seeds (Bacilio et al. 2003; Cassan et al. 2009a). It is reported that improved seed germination coincides with high GA production in *A. brasilense* cultures (Cassan et al. 2009b).

Azospirillum synthesizes and metabolizes GAs in vitro and in planta. The production of different GA compounds and metabolism of exogenously applied GA are reported for different *Azospirillum* species (Reis et al. 2011). Unlike IAA production, the mechanism of GA production is not known. However, dwarfism induced in maize plant by inhibitors of GA biosynthesis was

reverted by inoculation with *A. lipoferum* and *A. brasilense* (Lucangeli and Bottini 1997). Recently, Manivannan and Tholkappian (2013) isolated *Azospirillum* strains from tomato rhizosphere and estimated GA production from 3.3 to 9.5 µg/25 ml broth. GA₃ is the main GA identified in *Azospirillum*. Piccoli et al. (1999) reported the effect of water potential on GA₃ production. Water potential and oxygen concentration affect GA₃ production in *A. lipoferum*; production is reduced at high water potential and low O₂ concentration. Lucangeli and Bottini (1997) suggested the involvement of GA₃ produced by *Azospirillum* in growth promotion of maize.

Cytokinins

Cytokinins are derived from N-substituted amino-purines. In plants, these are produced in root tips and germinating seeds. These are transported in shoots to regulate cell division, shoot and root morphogenesis, chloroplast maturation, cell enlargement, leaf expansion, auxiliary bud release, and delaying senescence (Spaepen et al. 2009). Auxin is known to regulate the biosynthesis of cytokinin, and ratio of auxin to cytokinin is crucial during cell division and differentiation of plant tissues. Cytokinin production in defined culture medium has been reported for *Azospirillum* by several researchers (Tien et al. 1979; Horemans et al. 1986; Cacciari et al. 1989; Strzelczyk et al. 1994). Inoculation effect of these strains on plant is based on the balance between both the hormones produced by both the partners (Reis et al. 2011). Some *Bradyrhizobium* strains use cytokinins for nodulation (Giraud et al. 2007). *Azospirillum* being a cytokinin producer might support nodulation when co-inoculated with rhizobia as also reported earlier (Yahalom et al. 1990).

Abscisic Acid (ABA)

The main role of ABA is as a phytohormone produced in response to environmental stress, e.g., decreased soil water potential, heat, and water or salt stress. ABA produced in roots is translocated to leaves and alters osmotic potential of stomata guard cells leading to closing of stomata, preventing further water loss at the time of low water availability (Bartels and Sunkar 2005). ABA

production by *A. brasilense* Sp245 was reported by Cohen et al. (2008), but like GA compounds, production mechanism is not known.

Polyamines

Polyamines are low molecular weight organic compounds containing two or more primary amino groups. These compounds are synthesized in cell, but their exact function is not clear. However, if synthesis of these compounds is inhibited, cell growth is stopped or inhibited. Among polyamines, cadaverine is known to promote root growth and control stomata activity in plants (Bashan and de-Bashan 2010). *A. brasilense* Az39, a common inoculant for wheat and maize in Argentina, is known to produce polyamines including spermidine, spermine, putrescine in culture, and cadaverine in growth medium supplemented with precursor L-lysine and in rice plants inoculated with this strain (Thuler et al. 2003; Perrig et al. 2007). Bashan et al. (2004) proposed cadaverine as a contributing factor in whole plant response due to *Azospirillum* inoculation.

Ethylene

Ethylene is known for breaking seed dormancy and inhibition of root elongation if produced in higher amount. Ethylene level is lowered to prevent economic loss in agriculture and can be considered as having potential for promoting plant growth (Glick et al. 1999). 1-Aminocyclopropane-1-carboxylic acid (ACC) is a common precursor of ethylene, and ACC deaminase degrades this precursor. Although ACC deaminase is very common among PGPR, wild strains of *Azospirillum* spp. do not have this enzyme; nevertheless, some strains can produce ethylene (Perrig et al. 2007). However, ACC deaminase gene (*acdS*) was recently detected in *A. lipoferum* 4B, and a homolog of *acdS* gene has been identified in *Azospirillum* B510 genome (Pringent-Combaret et al. 2008; Kaneko et al. 2010).

Nitrogen Oxide (NO)

NO functions as a signal molecule in IAA-induced signaling cascade leading to adventitious root development (Pagnussat et al. 2002, 2003).

The role of NO in lateral root formation and root hair development is demonstrated by Correa-Aragunde et al. (2004) and Lombardo et al. (2006). *Azospirillum* is known to produce NO at low oxygen pressure by denitrification, since a long time ago (Hartmann and Zimmer 1994). Creus et al. (2005) quantified the production of 6.4 nmol NO/g of *A. brasilense* growing under aerobic conditions.

Phosphate Solubilization

Microbes have the ability to solubilize insoluble phosphate by producing enzymes such as phytases and phosphatases or organic acids. Enzymes help to release organic phosphorus, and organic acids release inorganic phosphorous. Around 40 % of culturable bacteria from soil have this ability (Reis et al. 2011). It is not common among species of *Azospirillum*. However, *Azospirillum* spp. can produce different organic acids that assist in phosphorus solubilization depending on sugars in root exudates. There are few reports available about *A. halopraeferans*, *A. lipoferum*, and *A. brasilense* on this. Gluconic acid production and pH reduction were observed in *A. lipoferum* JA4. For *A. brasilense* strains, pH reduction and gluconic acid production were observed when carbon source of the medium was replaced by glucose (Rodriguez et al. 2004). *A. halopraeferans* solubilized inorganic phosphorous (Seshadri et al. 2000). A recent report about phosphate solubilization by *A. brasilense* WS-1 was published by Tahir et al. (2013).

Siderophore Production

Siderophores are low molecular weight iron chelating compounds which help beneficial bacteria to take away the ferric ion and make it unavailable to pathogenic bacteria. It is considered as indirect growth mechanism by which PGPR promote plant growth. Catechol-type siderophore production by *A. lipoferum* and *A. brasilense* was reported earlier by Shah et al. (1992) and Tapia-Hernandez et al. (1990). Recently, *Azospirillum*

strains isolated from tomato rhizosphere had shown the production of catechol-type siderophores (Manivannan and Tholkappian 2013). Strains were not identified up to species level in this study. There are several reports for the siderophore production by *A. lipoferum* and *A. brasilense*. Boyer et al. (2008) detected siderophores in two strains TVV3 and B518 of *A. lipoferum*. Pedraza et al. (2007) isolated *A. brasilense* strains from strawberry plants and detected siderophore production by these strains. According to their observation, the amount of siderophore production has a correlation with its area of isolation, i.e., rhizosphere or within the plants. Endophytic strains produced higher amount.

Antimicrobial Activity

Oliveira and Drozdowicz (1987) reported bacteriocin production by *Azospirillum*. Somers et al. (2005) detected the production of Phenyl acetic acid (PAA) – an auxin-like molecule with antimicrobial activity, in culture supernatant, when phenylalanine was added as precursor. Tapia-Hernandez et al. (1990) isolated bacteriocin-producing *Azospirillum* strains that inhibited the growth of several other bacteria. In vitro HCN production by *Azospirillum* strains isolated from Brazil has been reported by Goncalves and de Oliveira (1998). Siderophores also exhibit antimicrobial activity against various bacterial and fungal isolates (Shah et al. 1992).

Azospirillum as a Biofertilizer

Plant Growth Promotion Due to Nitrogen Fixation

The oldest concept about plant growth promotion due to *Azospirillum* inoculation is based on its nitrogen-fixing ability. Although there are doubts about transfer of this fixed nitrogen to the plant, still several reports are available to support the contribution through BNF. A recent review by Okumura et al. (2013) discussed about BNF data for *Azospirillum*. The review is focused on five

crops and BNF contribution to these plants. Some of the examples selected from this review are described here. Brasil et al. (2005) inoculated a mixture of *A. brasilense* and *A. lipoferum* to *Brachiaria* plants and noticed higher root, shoot dry weights, and nitrogen content as compared to uninoculated plants. Kennedy et al. (2004) mentioned 26 % increase in corn productivity under field conditions. Saikia et al. (2007) suggested that high yield of *Azospirillum*-inoculated plants is due to higher photosynthesis and stomatal conductance in these plants. Okon and Vanderleyden (1997) reviewed 22-year field inoculation data of *Azospirillum* and concluded that this organism promotes crop productivity under different climates and soil conditions and goes beyond increase in BNF as increase in absorptive surface of root system was also observed. Sala et al. (2007) reported 26 % increase in wheat grain production when inoculated with *Azospirillum*. Diaz-Zorita and Fernandez-Canigia (2009) used liquid-based inoculants of *A. brasilense* for wheat, from 2002 to 2006, at 297 localities in Argentine Pampas region. The increase in productivity was 260 kg/ha and 70 % of grain yield. Several researchers are convinced that a combination of nitrogen fertilizer with *Azospirillum* inoculation is beneficial for wheat plants as inoculation promotes better absorption and utilization of available nitrogen. Dose of fertilizer recommended for combination varies from 15 to 160 kg N/ha (Saubidet et al. 2002; Dalla Santa et al. 2004; Sala et al. 2005).

Many studies showed that the contribution of nitrogen fixation by *Azospirillum* to the plant ranged from 5 to 18 % of total nitrogen increase in plant (Bashan and de-Bashan 2010). However, conclusive proof of the nitrogen contribution is based on ^{15}N dilution technique. Garcia de Salamone et al. (1997) used ^{15}N isotope dilution technique to determine the contribution of BNF to two genotypes of maize, inoculated with a mixture of *Azospirillum* strains. Significant increase in grain yield and total nitrogen content of both genotypes was observed. Saubidet and Barnex (1998) inoculated five cultivars of wheat with most efficient nitrogen-fixing strains of *A. brasilense*. Increase in growth and nitrogen con-

tent was observed, but response varied among cultivars. Didonet et al. (2003) inoculated ten lines of *Oryza sativa* with *A. brasilense* and *A. lipoferum* and reported that response depends upon cultivar and strains used. Mehnaz and Lazarovits (2006) reported significant increase in root and shoot dry weight of four corn varieties, inoculated with *A. zeae* (misidentified at that time as *A. lipoferum*); however, response varied with all varieties. Although *Azospirillum* is considered as a nonspecific PGPR with respect to host, there are several studies indicating that response varies among varieties and cultivars of the plants. Rodrigues et al. (2008) inoculated rice plants with *A. amazonense* and observed increased grain dry matter and nitrogen accumulation at maturation. Contribution from nitrogen fixation was up to 27 %. The comprehensive review by Bashan and de-Bashan (2010) based on the analysis of 35-year data suggested that even though there are controversial reports about nitrogen contribution to the plant due to nitrogen fixation by *Azospirillum*, the mechanism cannot be ignored.

Plant Growth Promotion due to Phytohormone Production

When plants are inoculated with bacteria, root system is the first plant part which shows response. Inoculation increases number and length of root hairs, number of lateral roots, diameter and length of lateral roots and adventitious roots, and root surface area. The increased root system can improve plant nutrition and in general is responsible for plant growth promotion. Change in the root system is considered due to the production of auxins, and *Azospirillum* is well known for these compounds. El-Khawas and Adachi (1999) inoculated rice plants growing in hydroponic system, with filter-sterilized supernatants of *Azospirillum* culture. Increase in root dry weight, surface area, lateral roots, root hairs, and root elongation as compared to uninoculated plants was observed. Cell-free supernatant of *A. brasilense* Cd was applied to soybean plants, and increase in root numbers and length was observed (Molla et al. 2001). In contrast, wheat inoculation

with *A. brasilense* Sp 245 and Sp7 decreased root length; however, root hair formation increased (Dobbelaere et al. 1999). This type of effect on root system is very common when higher concentrations of IAA are applied exogenously. Remans et al. (2008) proved that response of exogenously applied IAA on bean roots resembled to *Azospirillum* inoculation. Malhotra and Srivastava (2006) engineered *A. brasilense* SM strain and made it to produce threefold higher IAA as compared to wild-type strain. *Sorghum* plants inoculated with this strain showed higher effect on lateral branching and dry weight of roots when compared with plants inoculated with wild type. Although there are several reports of plant growth promotion due to IAA production by *Azospirillum*, considering it as a single mechanism responsible for growth promotion needs more evidences.

Cohen et al. (2009) inoculated *A. lipoferum* in maize plants which were unable to produce ABA and GA, due to application of inhibitors of biosynthetic pathways of these compounds. It causes the effect similar to drought; however, inoculation with *A. lipoferum* reversed the effect which suggested the participation of ABA and GA in alleviating water stress in the presence of the bacteria. So far, several researchers are convinced that water stress alleviation of plants by *Azospirillum* involves the production of ABA, auxins, and GAs (Costacurta and Vanderleyden 1995; Bottini et al. 2004; Cohen et al. 2008).

Plant Growth Promotion Due to Phosphate Solubilization

Among 15 species of *Azospirillum*, *A. halopraeferens* can solubilize insoluble inorganic phosphate in vitro by unknown mechanism as it does not use glucose, and therefore does not produce gluconic acid (Seshadri et al. 2000). Some strains of *A. brasilense* and *A. lipoferum* are capable of producing gluconic acid and can solubilize rock phosphate (Puente et al. 2004; Rodriguez et al. 2004). Chang and Li (1998) isolated *Azospirillum* strains from ectomycorrhizal sporocarp which degraded limestone, marble, and calcium phos-

phate in vitro. Carrillo et al. (2002) inoculated cardon (cactus) with *A. brasilense* Cd strain and reported enhanced phosphate solubilization and plant growth. Deubel et al. (2000) inoculated pea plants with *Azospirillum* strains and observed a shift in sugars under phosphorous deficiency which increased the capability of *Azospirillum* spp. to mobilize phosphate. Sugars such as glucose are part of pea root exudates grown in phosphorous-deficient soils, thus increasing the capacity of *Azospirillum* spp. to solubilize insoluble $\text{Ca}_3(\text{PO}_4)_2$. It was observed that under phosphorous deficiency glucose content decreased, while galactose, ribose, xylose, and fructose increased in root exudates.

Plant Growth Promotion by Minimizing the Stresses

The use of *Azospirillum* in addition to other PGPR to alleviate the salt stress and minimize the negative effects of several plant stresses including water stress has been recommended by several researchers (Bacilio et al. 2004; Bashan et al. 2004; Lucy et al. 2004). Salt resistance among *Azospirillum* species increased from *A. amazonense* (lowest) to *A. halopraeferans* (highest), as the latter tolerates over 3 % that is equivalent to seawater salinity (Bashan and de-Bashan 2010). *A. brasilense*-inoculated wheat plants showed a significant increase in shoot elongation, fresh and dry weights, water content, and relative water content in shoots, when grown under 320 mM NaCl and 20 or 30 % polyethylene glycol (PEG) 6000 causing salt or osmotic stress, respectively (Creus et al. 1997). Barassi et al. (2006) used *A. brasilense* Sp245 to inoculate lettuce seeds and observed the effect of salt stress. Higher total fresh and dry weights were recorded as compared to uninoculated plants. Inoculated dried seeds maintained the ability to mitigate the effect of salt on lettuce germination. The same strain alleviated the salt stress in carrot and chickpea as well (Hamaoui et al. 2001; Ayrault 2002). Rueda-Puente et al. (2004) suggested the use of *Klebsiella pneumoniae* and *A. halopraeferans* as auxiliary biofertilizers to improve the growth of *Salicornia bigelovii* (halophyte) in coastal

semiarid zones. Later on, the same group reported that yield loss due to stress diminished in *Azospirillum*-inoculated wheat plants, and grains showed 38.4, 22.2, and 125 % higher Mg, K, and Ca, respectively, as compared to uninoculated ones (Creus et al. 2004).

Inoculation with *Azospirillum* improved growth under water stress conditions, which was initially demonstrated in the 1980s (Bashan and Levanony 1990). Okon (1985) stressed in a review about the importance of *Azospirillum* in improving water status of the plants. It suggests that *Azospirillum* inoculation can protect the crops against drought. Sarig et al. (1990) inoculated *Sorghum bicolor* plants to osmotic stress in hydroponic system and observed that due to *A. brasilense* inoculation adverse effects such as reduction in leaf senescence were diminished. Corn plants inoculated with *Azospirillum* showed less effect of water stress on plant growth, as increase in root growth, total aerial biomass, foliar area, and proline content in leaves and roots was observed (Casanovas et al. 2002). *Azospirillum* inoculation alleviated the stress on wheat plants grown under drought conditions (El-Komy et al. 2003). Creus et al. (2004) inoculated wheat seeds with *A. brasilense* Sp245 at flowering stage and subjected the plants to drought. Significantly higher water content, relative water content, water potential, apoplastic water fraction, lower cell wall modulus of elasticity values, reduction in grain yield loss, and significantly higher Mg, K, and Ca in grains were observed in inoculated plants. Moutia et al. (2010) inoculated two cultivars of sugarcane with *Azospirillum* sp. strains under drought and non-drought conditions. Both cultivars showed different results as one showed 15 % increase in shoot height and 75 % increase in dry weight under drought, whereas the other cultivar showed negative results under non-drought conditions. Kasim et al. (2013) used *A. brasilense* NO40 to prime the wheat seeds and grew them under drought conditions. Priming significantly alleviated the deleterious effect of drought stress on wheat. Inoculated plants showed attenuated transcript level suggesting improved homeostatic mechanism due to priming.

Rodriguez-Salazar et al. (2009) used an engineered strain of *A. brasilense* with the ability to produce trehalose (osmotic regulating sugar), more salt tolerant than wild type, to inoculate maize plants grown under stress. Inoculation significantly enhanced survival of these plants under water stress. According to Bashan and de-Bashan (2010), limitations regarding the effect of inoculation under saline stress are valid for osmotic stress.

There are few reports about decreasing the toxic effect of metals on plants due to *Azospirillum* inoculation. Cadmium (Cd) inhibits the growth and nutrient uptake in barley plants. *A. lipoferum-inoculated* barley plants showed decreased toxicity to Cd, enhanced root length and biomass, and significant increase in amount of nutrients absorbed (Belimov and Dietz 2000; Belimov et al. 2004). Lyubun et al. (2006) observed less accumulation of arsenic in *A. brasilense* Sp245-inoculated wheat plants as compared to uninoculated plants. The association of *Azospirillum* changed the speciation, bioavailability, and uptake of arsenic by plants.

There are individual reports about positive effect of *Azospirillum* inoculation on plants growing under different stresses such as pH, toxic substances, high light intensities, humic acid, etc. High pH decreases the microalgal population. *A. brasilense* inoculation in microalgal culture eliminated the negative effect (de-Bashan et al. 2005). High level of tryptophan reduced multiplication of *Chlorella vulgaris*, but co-culturing with *A. brasilense* significantly reduced the inhibition (de-Bashan and Bashan 2008). Sometimes light intensity has negative effect on crops. Wheat inoculation with *A. brasilense* Cd significantly increased quantity of photosynthetic pigments chlorophyll a, b and auxiliary photoprotective pigments violaxanthin, zeaxanthin, antheraxanthin, lutein, neoxanthin, and β -carotene that help the plant to sustain photosynthesis under unfavorable light conditions (Bashan et al. 2006). Hernandez et al. (2006) used *A. brasilense* to inoculate *Chlorella* spp. that is used for wastewater treatment and observed enhanced growth and metabolism of the microalgae. Bacilio et al. (2003) inoculated wheat seeds

with *A. brasilense* or *A. lipoferum* and observed improved seed germination and plant growth in soils amended with compost containing high concentration of humic acid. Both bacterial species survived in high humic acid solutions and modified compost composition “in vitro.”

Considering the reports about *Azospirillum* effect on plant growth under different stresses, it is clear that several known and unknown mechanisms are involved in the interaction of this organism with plants. Based on these findings, Cassan et al. (2009b) and Sgroy et al. (2009) proposed a term “plant stress homeo-regulating bacteria (PSHRB)” for all rhizosphere PGPR/PGPB including *Azospirillum* that regulate homeostasis in plants under abiotic stresses.

Miscellaneous Effects of *Azospirillum* on Plants

Azospirillum inoculation in maize plants led to major quantitative and qualitative modification of secondary metabolites especially benzoxazinoids (Walker et al. 2011). *Azospirillum* produces nitric oxide (NO) which participates in metabolic, signaling, defense, and developmental pathways in plants and can also modify root architecture (Molina-Favero et al. 2007, 2008). *Azospirillum* can produce nitrite as part of its normal metabolism which also participates in plant growth promotion (Zimmer et al. 1988). Nitrate reductase (NR) activity of wheat leaves decreased by *Azospirillum* inoculation and increased in nitrate assimilation was also reported (Ferreira et al. 1987). Some of the lectins produced by *Azospirillum* induced changes in mitotic state of growing onion plant cells (Nikitina et al. 2004). Cell surface lectins isolated from *A. brasilense* affected activities of α -glucosidase, β -glucosidase, and β -galactosidase in the membrane and apoplast fractions of roots of wheat seedlings (Alenkina et al. 2006). *A. brasilense* Cd inoculation enhanced the proton efflux of wheat root (Bashan 1990). Inoculation of soybean seedlings with the same strain significantly reduced the membrane potential in every root part (Bashan 1991; Bashan and Levanony 1991).

Bashan et al. (1992) proved the effect of *A. brasilense* on root cell membrane of soybean and cowpea plants. Pereyra et al. (2006) showed that fatty acid composition of main phospholipids in roots is affected by *A. brasilense* inoculation. *A. lipoferum* 34H increased the phosphate ion content, resulting in significant increase in root length and fresh and dry weight of shoots of rice plants (Rodriguez and Fraga 1999). During dry storage, seeds deteriorate and germination gets delayed. Lettuce seeds inoculated with *A. brasilense* along or after an osmo-priming treatment could mitigate the negative effect of ageing (Carrozzi 2005).

Azospirillum as a Biocontrol Agent

The role of *Azospirillum* as a biocontrol agent has always been ignored, although reports for its anti-pathogenic activity were available since a long time ago. Mechanisms such as environmental competition, displacement of pathogens, inhibition of seed germination of parasitic weeds, inhibition of fungal growth via the production of toxic substances, and growth enhancement of plants to resist pathogen are suggested for *Azospirillum* to play its role as a biocontrol agent (Bashan and de-Bashan 2010).

As an Antimicrobial Agent

When *A. brasilense* Cd was co-cultured with pathogenic *Staphylococcus* spp., population of pathogenic bacteria got reduced (Holguin and Bashan 1996). Mixed cultures of *A. brasilense* and tomato pathogen *Pseudomonas syringae* were inoculated on tomato seeds. Significant decrease in severity of disease and improved plant growth was observed as *P. syringae* did not survive in the presence of *A. brasilense* (Bashan and de-Bashan 2002). *A. brasilense* reduced the dry weight of mycelium of four soilborne fungal pathogens of cucumber, i.e., *Fusarium* (90–96 %), *Rhizoctonia* (72–94 %), *Pythium* (71–95 %), and *Sclerotinia* (100 %), during an in vitro study (Hassouna et al. 1998). Sudhakar et al. (2000)

applied *A. brasilense* as foliar spray against foliar fungal and bacterial diseases of mulberry. Inoculation reduced fungal pathogens and excelled as a treatment against bacterial blight. Co-inoculation of *Azospirillum* spp. with arbuscular mycorrhizal (AM) suppressed damping off disease in *Capsicum* sp., caused by *Pythium aphanidermatum* (Kavitha et al. 2003). Tortora et al. (2011) isolated *A. brasilense* strains with the ability to produce catechol-type siderophores including salicylic acid. Inoculation of these strains reduced the anthracnose disease caused by *Colletotrichum acutatum* in strawberry plants.

As a Pesticide

Inoculation of *A. brasilense* to *Sorghum* resulted in tenfold decrease of dead heart disease caused by a shoot fly *Atherigona soccata* and also resulted in increase in grain yield (Kishore 1998). Only a few root galls and egg masses per root system, of mung bean plants infected with root-knot nematode, were observed when inoculated with *A. lipoferum* (Khan and Kounsar 2000). The increase in pod yield and reduction in root-knot nematode egg masses and total nematode population was observed in Okra plants, inoculated with *A. brasilense* (Ramakrishnan et al. 1997). A combination of mycorrhizal fungi, phosphate-solubilizing bacteria, and *Azospirillum* reduced the population of *Pratylenchus zeae* (nematode) and increased the cob yield of maize (Babu et al. 1998). *A. lipoferum* inoculation in wheat plants reduced *Heterodera avenae* (nematode) infection (Bansal et al. 1999).

As a Herbicide

Azospirillum spp. inhibited germination of a parasitic weed, *Striga hermonthica* (witch weed), of *Sorghum* fields (Bouillant et al. 1997). Lipophilic compounds produced by *A. brasilense* in culture medium inhibited the germination of *Striga* seeds (Miché et al. 2000). *A. brasilense* inhibited germination and radicle growth of *Orobanche aegyptiaca* (Egyptian broomrape) seeds, a parasitic weed of sunflower (Dadon et al. 2004).

As an Elicitor of Induced Systemic Resistance

The role of rhizobacterial exopolysaccharides (EPS) as elicitor of induced systemic resistance (ISR) was reported by Kyungseok et al. (2008). Recently, Sankari et al. (2011) used different concentrations of EPS produced by *Azospirillum* strains as inoculants of rice plants. EPS-inoculated plants showed better growth and reduction in blast disease caused by *Pyricularia oryzae*. *Azospirillum* sp. B510 induced disease resistance in rice plants against *Xanthomonas oryzae* and fungus *Magnaporthe oryzae*. Resistance was due to a novel mechanism independent of salicylic acid-mediated defense (Yasuda et al. 2009).

Azospirillum as a Commercial Biofertilizer

Azospirillum is capable of colonizing vegetables, cereals, and several other plants and induces beneficial effects as it congregates several plant growth-promoting mechanisms which make it a valuable candidate as a biofertilizer. For more than 40 years, researchers are using *Azospirillum* as an inoculant in lab, pots, and field experiments. Several reviews are available which have covered the 20–35-year data based on growth promotion by different strains of this genus. All of the researchers and reviewers agree on one point that this bacterium is beneficial for plant, and there is not a single report about its harmful effect. However, there is also an agreement that plant growth promotion is not due to any single mechanism; rather, it is a combination of several mechanisms possessed by *Azospirillum*, and in a recent review Bashan and de-Bashan (2010) proposed it as “multiple mechanisms theory.” According to this theory “there is no single mechanism involved in promotion of plant growth by *Azospirillum* but a combination of a few or many mechanisms in each case of inoculation.” These may vary according to plant species, *Azospirillum* strain, and environmental conditions when interaction occurred. Actually, possession of several mechanisms makes this organism one of the best candidates for bioinoculants.

Different countries have launched the *Azospirillum* as a biofertilizer for different crops. Information given below is based mainly on Reis et al. (2011) and Internet search:

1. *Azo-Green*TM: A product of Genesis Turfs Forages Company, based in the USA, contains only *A. brasilense*. Recommended usage is as seed coat to improve seed germination, root system, drought resistance, and plant health.
2. *Zea-Nit*TM: A product of Heligenetics Company, formulated in Italy, Germany, and Belgium, contains a mixture of *A. lipoferum* Br17 and *A. brasilense* Cd. It is sold as liquid formulation or in a mixture of vermiculite. Company recommended the usage of 30–40 % less nitrogen fertilizer with inoculum.
3. *Azo-Green*TM: A French company launched a biofertilizer under the same name as used by the previously mentioned US company. However, this inoculant is specific for maize and contains *A. lipoferum* CRT-1. Based on field experiments carried out in Africa, 100 % increase in yield was claimed.
4. *Fertilizer for Maize*: A product developed by University of Puebla, Mexico, in 1993.
5. *Inoculant for coffee plants*: In 2008, *Azospirillum* inoculant was developed for coffee plants in Mexico.
6. *Graminante*TM: A product of Uruguay contains bacterial inoculum in powder form, mixed with calcium carbonate.
7. *Inoculant for maize and rice*: In 2009, a company in Brazil started selling a biofertilizer based on *Azospirillum* strains for maize and rice.
8. *Inoculant for cereals and sunflower*: Several companies of Argentina are selling biofertilizers based on *A. brasilense* for cereal crops, sunflower, etc.
9. *Gmax Nitromax*: A product of GreenMax AgroTech, India, containing a mixture of *Azospirillum* and *Azotobacter*, available for all crops except legumes. There are several companies in India which are selling biofertilizers containing single or combinations of different microbes including PGPR and fungi.



Fig. 15.4 A biofertilizer of *Azospirillum* for wheat crop, commercially available in Pakistan

10. *Bio N-Plus*: A product of Pakistan contains *A. lipoferum*.
11. *BioPower*: A product of Pakistan contains a mixture of PGPR including *A. brasilense* and *A. lipoferum* (Fig. 15.4).

Conclusion

Bashan and de-Bashan (2010) concluded three facts about *Azospirillum*: (1) Most *Azospirillum* strains can fix nitrogen but only a fraction of it is transferred to the plant; (2) many strains produce phytohormones “in vitro” and some in association with plants, but transfer is probably limited; and (3) a general positive growth response in numerous plant species is evident in majority cases, but effect is not always apparent in terms of economic productivity. If we look at these points, it reflects that although authors have suspicions about the contribution of nitrogen fixation and phytohormone in health of inoculated plants, they also report that they are convinced that inoculation does end up in plant growth promotion even if it is not always in terms of yield

increase. I would suggest that researchers should consider the importance of parameters other than yield increase because if we look from a farmer's point of view, then his concern is the plant growth, and *Azospirillum* does that by all means. However, from a researcher's point of view, it is important to find evidence of the claimed mechanisms which means a more comprehensive study of plant microbe interactions.

Going through the data available since *Azospirillum* was isolated for the first time, it is very clear that most of it is based on inoculation of *A. lipoferum* and *A. brasilense*. Similarly, if we look into details of *Azospirillum* biofertilizers available all over the world, these products also contain either *A. lipoferum* or *A. brasilense*, individually or together. Considering these facts:

- (a) It is suggested that researchers should start exploring the rest of the 13 species for their potential as biofertilizers.
- (b) As the importance of *Azospirillum* as a bio-control agent has been always ignored, now it is time to focus on this aspect and study it as extensively as its role as a biofertilizer has been evaluated.
- (c) Although several companies are selling *Azospirillum* as a biofertilizer, still it is not as popular as *Rhizobium* biofertilizer. The main reason is variable response of this organism with different crops which suggests that extensive optimization studies for the inoculum amount with reference of specific crops are still required.
- (d) It is suggested that optimization studies should also be performed for different cultivars and varieties as enough reports are present to support that inoculation response varies with genotype of the same host.

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