Chapter 6
What Is Expected from the Genus *Azospirillum* as a Plant Growth-Promoting Bacteria?

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6.1 The Genus *Azospirillum*

The discovery of the first species of microaerophilic nitrogen-fixing bacteria of the genus *Azospirillum* was due to the introduction of nitrogen-free semisolid media. Under the conditions of oxygen gradient formed in semisolid media, the organisms, attracted by their characteristic aerotaxis, move to the region within this medium where their respiration rate is in equilibrium with the oxygen diffusion rate. They form typical veil-like pellicles approximately 5 mm below the surface, and then they move up close to the surface when, due to their N_2-dependent growth, more and more cells accumulate. After this discovery, several other diazotrophs with the same microaerophilic behavior were also isolated, belonging to other bacterial genera but using the same strategy.

At least 15 *Azospirillum* species have been described, but in terms of physiology and genetics the most studied ones are *A. lipoferum* and *A. brasilense* described by Tarrand et al. (1978). Both are abundant, mainly in tropical areas, associated with forage grasses, maize, wheat, rice, sorghum, sugarcane, and several other plants that also harbor this bacterial genus (Hartman and Baldani 2006; Zambrano et al. 2007). However, besides its association with plants, *Azospirillum* species have also been associated with other environments under extreme conditions of temperature or contamination (Nosko et al. 1994; Eckford et al. 2002; Young et al. 2008). The third species *A. amazonense* was isolated and described in the year 1983 from forage grasses planted in Amazonian region but is also associated with rice,

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maize and sorghum rhizosphere, and other grasses in the central-south of Brazil (Magalhães et al. 1983; Reis Jr et al. 2006). A group in Germany classified *A. halopraeferans*. It was isolated from kallar grass (*Leptochloa fusca*) growing under saline conditions in Pakistan and seems to be specific to that plant, since attempts to isolate *A. halopraeferans* from other plants growing in saline soil in Brazil failed (Reinhold et al. 1987, 1988). Further on, a new species isolated from rice plants in Iraq was described and named *A. irakense* (Khammas et al. 1989), although subsequently this species has not been reported as isolated from other grasses or any other country its name was validated as *Azospirillum* new species (List No. 39, 1991). In 1997, studies performed in Australia, using analysis of the 16S rDNA sequence of *Conglomeromonas largomobilis* subsp. *largomobilis*, showed that this bacterium had close relation to the species *A. lipoferum* and *A. brasilense*, but sufficiently distant to guarantee separate species status. On the basis of the phylogenetic evidence, Ben Dekhil et al. (1997) proposed the transfer of the subspecies *C. largomobilis* ssp. *largomobilis* to the genus *Azospirillum* as *Azospirillum largomobile* that was then corrected to *A. largimobile* (Sly and Stackebrandt 1999). New group of *Azospirillum* species continued to be described all around the world. In 2001, it was described and named *A. dobereinerae* in honor to the Brazilian scientist Johanna Döbereiner who initiated the studies of this genus in Brazil (Eckert et al. 2001). Another species was isolated from the paddy soil of a rice plant in 1982 and named as *A. oryzae* in China (Xie and Yokota 2005). Then, using a forage grass planted in China, another species was also described using surface sterilized roots and stems of *Melinis minutiflora* Beauv, and in this case, several modified semisolid media were used to collect 15 new strains of *A. melinis* (Peng et al. 2006). In 2007, using the same medium at pH 7.2–7.4 described by Xie and Yokota (2005), two new strains were identified by a research scientist group from Canada. These isolates were obtained from the rhizosphere of corn planted in Ontario and were named *A. canadense* (Mehnaz et al. 2007a) and *A. zeae* (Mehnaz et al. 2007b). *A. canadense* utilizes other carbon sources not used as the discriminative ones among previously described species as presented in Table 6.1. It uses many organic acids such as malate, pyruvic, acetic, succinic, citric, and formic acids. A newly described *Azospirillum* species was isolated from oil-contaminated soil by a group in Taiwan using nutrient agar. It was named *A. rugosum* as they form colonies that changed to wrinkled appearance on solid agar-medium and could be differentiated from its close phylogenetic relatives (*A. canadense, A. brasilense*, and *A. doebereinerae*) on the basis of carbon source utilization, gelatin hydrolysis, nitrate reduction, and arginine dihydrolase activity (Young et al. 2008). In 2009, two new species were described: *A. palatum* (Zhou et al. 2009) and *A. picis* (Lin et al. 2009). The first one was isolated from soil in China and according to the description is negative for acetylene reduction, do not produce indoles or reduce nitrate and/or nitrite. The second one was from Taiwan from discarded road tar, which generally contains, among other things, polycyclic aromatic hydrocarbons (PAH) and other poisonous or carcinogenic compounds. The *A. picis* fix nitrogen and is also positive for nitrate reductase, but indoles production has not been observed. Most recently another new species have been proposed, *Azospirillum*

Table 6.1 *Azospirillum* species and their pattern of carbon sources utilization

Azospirillum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A. lipoferum	+	+	−	+	v	+	−	v	+	+	v	−	−	+	v	−	+	+	−	−
A. brasilense	−	v	−	+	−	v	−	+	v	+	−	−	−	−	−	−	−	−	−	−
A. amazonense	v	+	+	+	+	+	+	−	+	−	+	v	+	−	+	v	+	−	+	+
A. halopraeferans	nd	v	nd	+	nd	−	nd	nd	−	+	nd	nd	nd	+	+	nd	+	−	−	nd
A. irakense	+	+	+	v	+	+	+	−	+	−	−	+	+	−	+	+	v	−	+	+
A. largimobile	+	+	−	+	−	+	+	−	+	+	−	−	−	+	−	−	+	+	nd	−
A. dobereinerae	−	nd	−	+	nd	v	−	v	v	+	−	−	−	+	−	−	−	+	−	−
A. oryzae	nd	+	−	+	nd	+	nd	nd	+	nd	−	−	−	−	nd	+	+	−	−	nd
A. melinis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	+
A. canadense	−	−	−	−	−	−	−	nd	−	−	−	−	−	−	−	−	nd	−	−	−
A. zeae	v	+	−	+	+	+	−	nd	v	+	−	−	−	+	−	−	nd	+	−	−
A. rugosum	−	−	nd	+	nd	nd	nd	+	+	nd	nd	−	−	−	nd	nd	nd	nd	nd	nd
A. palatum	v	v	+	+	nd	nd	nd	nd	+	−	nd	nd	+	+	nd	nd	nd	−	+	+
A. picis	+	+	−	nd	−	+	−	+	+	nd	nd	nd	−	+	−	−	+	+	−	−
A. thiophilum	nd	+	nd	+	nd	+	nd	nd	+	+	nd	−	nd	+	−	−	nd	+	−	−

Carbon sources: 1. N-acetylglucosamine, 2. L-arabinose, 3. D-cellobiose, 4. D-fructose, 5. L-fucose, 6. D-galactose, 7. Gentiobiose, 8. D-gluconate, 9. D-glucose, 10. Glycerol, 11. Myo-inositol, 12. Lactose, 13. Maltose, 14. D-mannitol, 15. D-mannose, 16. L-rhamnose, 17. D-ribose, 18. D-sorbitol, 19. Sucrose, 20. D-trehalose

Data from Eckert et al. (2001), Sly and Stackebrandt (1999), Ben Dekhil et al. (1997), Khammas et al. (1989), Reinhold et al. (1987), Xie and Yokota (2005), Peng et al. (2006), Mehnaz et al. (2007a, b) and Lavrinenko et al. (2010)

Symbols: +, positive; −, negative; v, variable or inconsistent; nd, not determined

thiophilum, isolated from sulfur bacterial mat collected from a sulfide spring in Russia (Lavrinenko et al. 2010). Although it has close relationship with several *Azospirillum* species, this new isolated strain BV-ST is capable of mixotrophic growth under microaerobic conditions with the simultaneous utilization of organic substrates and thiosulfate as electron donor for energy conservation. To summarize the knowledge accumulated about the genus up to now, we can assume that *Azospirillum* spp. are Gram-negative bacteria that belong to the alphaproteobacteria phylum. On the basis of the newly discovered species, they are present in a wide diversity of environments and plants, including not only those of agronomic importance such as cereals, sugarcane, and forage grasses, but also from other plant species such as coffee and fruit plants and orchids. They are aerobic nonfermentative chemoorganotrophs, vibroid and produce several phytohormones, mainly auxins (not described for all species yet). They utilize several carbon sources mainly sugars and sugar alcohols (Table 6.1) and the pattern of carbon utilization has been used for discriminatory purpose among species of the genus.

6.2 Genetics and Biochemistry

Data acquired from more than 30 years of the *Azospirillum* genetic and biochemical traits have been extensively reviewed by Steenhoudt and Vanderleyden (2000). Most structural and regulatory genes related to nitrogen-fixation genes including

nod genes have been identified in *Azospirillum* spp.; however, the mechanism of their regulation at transcriptional and translational levels is still a matter of study. Several research groups have dedicated to study the mechanisms of action of Ntr system, NifA, PII, and several other genes involved in the central nitrogen metabolism of *Azospirillum* (Araújo et al. 2004, 2008; Huergo et al. 2008).

Genes involved in chemotaxis signal transduction in other bacterial species (c*heA,W, Y, B*, and *R*) were identified in *Azospirillum* and most recently a *chsA* -Tn5 mutant strain (74031) isogenic to the *A. brasilense* wild type Sp7 was not impaired in its growth rate but showed reduced motility in soft-agar plates (Carreño-López et al. 2009). The authors observed that the impairment of motility during chemotaxis was not related to alteration of flagella synthesis but to different responses to chemotactic compounds. The deduced protein ChsA contains a sensory PAS domain and an EAL domain suggesting it may be part of the signaling and regulation system of another pathway controlling chemotaxis in *Azospirillum*. This data may reinforce the presence of multiple chemotaxis systems in this genus as previously reported in the literature.

The genome of *Azospirillum* sp B510, an isolate from inner tissue or rice plants, consisted of a single chromosome (~3.311 Mbp) and six plasmids designated as: pAB510a, pAB510b, pAB510c, pAB510d, pAB510e, and pAB510f. The total of 3,416 protein-encoding genes is distributed among the genome and most of the plasmids, but 25% (1,559) have been assigned as hypothetical genes. The high number of rRNA genes, nine sets of *rrns* genes, is also a peculiar characteristic of this species that is not typical of alphaproteobacteria genome, studied so far (Kaneko et al. 2010). Genes other than the *nif* gene cluster that are involved in N_2 fixation and are homologs of *Bradyrhizobium japonicum* USDA110 include *fixABCX, fixNOQP, fixHIS, fixG*, and *fixLJK,* present in the genome of this bacterium. In *A. brasilense*, IAA is generally synthesized from tryptophan via indole-3-pyruvic acid (IPyA), and genes coding enzymes related to this pathway are cotranscribed in this species. Interestingly, no homolog of the *ipdC*, which codes for the key enzyme indole-3-pyruvate decarboxylase, or *iaaC* was found in the B510 genome. Otherwise, two putative plant hormone-related genes encoding tryptophan 2-monooxytenase (*iaaM*) and indole-3-acetaldehyde hydrolase (*iaaH*), homologous to genes of *A. tumefaciens* and *P. syringae*, could play this role in IAA biosynthesis via indole-3-acetamide (IAM) pathway. Another relevant data is that in *Azospirillum* sp. B510 genome a homolog of *acdS* gene was identified, although it was previously reported that plant growth promotion (PGP) trait related to ACC (amino cyclopropene carboxylic acid) deaminase activity was absent in *A. brasilense* (Holguin and Glick 2003). Comparison between the *Azospirillum brasilense* Sp7 pRhico (plasmid that is responsible for the interaction with plant roots) and pAB510f suggests an evolutionary link and some functional relationship between both. However, remaining portions of pRhico, such as those containing exoC and tRNA genes, were scattered throughout the B510 genome and most of the sequences were poorly conserved, indicating that drastic genome rearrangements had taken place since the two species diverged as posed by Kaneko et al. (2010). During this century the description of new species and the recent publication of the

genome of an *Azospirillum* sp. associated with rice brought new insights into the genetic potential of this genus, not only to agricultural application but also to bioremediation and biocontrol (Bashan et al. 2004; Kaneko et al. 2010).

6.3 Bacterial Behavior in Plants

The associated bacteria capable of stimulating plant growth are generally known as PGPR mainly because of their ability to reduce other bacterial population and competence to establish the association to plant roots (Kloepper and Schroth 1981). However, this term can introduce some bias in its application since not only root-associated bacteria can contribute to PGP mechanisms as also posed by the authors. Thus, in order to include other beneficial plant–bacteria associations besides the root-associated we adopt in this chapter the term plant growth-promoting bacteria (PGPB) according to the definition found in the study by Bashan and Bashan (2005).

Bacterial colonization is well described for *Azospirillum*, especially *A. brasilense*. Attachment to the root system is mediated by the polar flagellum and is followed by an irreversible anchoring of the bacteria after a period of time (Steenhoudt and Vanderleyden 2000). Figure 6.1 shows the biphasic model of the colonization proposed by Steenhoudt and Vanderleyden (2000), but in this case other phenomenon exists with a homologous local strain of *A. brasilense* isolated in Argentina, associated with strawberry roots (Pedraza et al. 2007).

The lateral flagella are not essential during the adsorption phase for the colonization process. But how is the behavior of a population on the root system? Is quorum sensing (QS) involved in this coordinative process? The QS was previously shown to regulate swarming motility in diverse bacteria, notably in *Serratia*

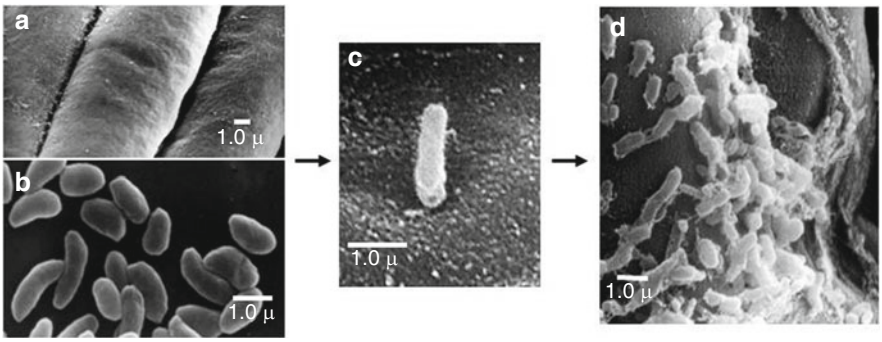

Fig. 6.1 Biphasic attachment process of *A. brasilense* to the root surface. (**a**) Strawberry root surface without inoculation; (**b**) Pure culture of *A. brasilense* REC3; (**c**) First step, *Azospirillum* as a single cell attached to the root surface; (**d**) Second step, bacterial cells forming aggregates and imbibed within the fibrillar material

marcescens (Eberl et al. 1996) and *Yersinia enterocolitica* (Atkinson et al. 2006). Boyer et al. (2008) tested two *A. lipoferum* strains, TVV3 and B518, isolated respectively from the rice rhizosphere (cultivar IR64) in Vietnam and from disinfected stems of rice (cultivar Kasalath) in Japan. In strain TVV3 *luxI* and *luxR* homologs have been identified but could not be inactivated, while strain B518 (isolated as a rice endophyte) produced larger amount of N-acylhomoserine lactones (AHLs) than did the strain TVV3. In order to quench AHLs accumulation in *A. lipoferum*, the *attM* gene from *Agrobacterium tumefaciens*, encoding an AHL-lactonase, was cloned under the constitutive PnptII promoter and conjugated into *A. lipoferum*. The results showed that in *A. lipoferum*, swarming and swimming motilities on semisolid agar plates were not affected by AHL inactivation. However, synthesis of Laf, the main structural component of the lateral flagella responsible for swarming motility in *A. lipoferum*, is impaired in B518 (pBBR1-*attM*), as revealed by proteomic analysis (Boyer et al. 2008). Thus unlike what occurs in a *laf*1 mutant of *A. brasilense* Sp7 that has lost its ability to swarm (Moens et al. 1995), B518 retains its ability to swarm despite the absence of Laf; this could be explained by the putative QS-independent production of biosurfactants implicated in bacterial swarming, as serrawetin in *S. marcescens* (Lindum et al. 1998).

But, is QS a common feature in the *Azospirillum* genus? Vial et al. (2006) used several strains of *Azospirillum* in order to verify if the common molecule of QS is present in this genus. AHL-mediated quorum-sensing in *Azospirillum* appears to be an exception rather than a rule. The authors tested 40 *Azospirillum* strains for their ability to synthesize AHLs. The AHL production was detected in four strains belonging to the *A. lipoferum* species isolated from rice rhizosphere. The authors also imply that other molecules not yet discovered can play a role in this genus, such as 3-hydroxypalmitic acid methyl ester (Clough et al. 1997) as observed in *Ralstonia*, or cis-11-methyl-2-dodecenoic acid described in *Xanthomonas* (Wang et al. 2004), two genera belonging to the alphaproteobacteria phylum as well.

The fact that AHLs are not implicated in regulation of phytostimulatory effects of *A. lipoferum* is an interesting result, as many bacteria possessing AHL-deactivating enzymes are rhizosphere inhabitants (Uroz et al. 2003). Moreover, QS in the rhizosphere can also be disrupted by abiotic factors such as alkaline pH, and by biotic factors, such as AHL mimics produced by some plants (Teplitski et al. 2000).

Bacteria colonize the rhizoplane and are found in high number upon emergence of lateral roots and also near the root cap (de Oliveira et al. 2002). These sites are normally colonized by several bacteria as they exude more carbon sources than other root areas, as they are growing regions. *Azospirillum* as a motile bacterium is capable of navigating in gradients of oxygen, redox molecules and nutrients by constantly monitoring its environmental changes in order to inhabit where it is optimal for surviving and growing. Although there is no strict host specificity in *Azospirillum*–plant associations, a strain-specific chemotaxis was reported; strains isolated from the rhizosphere of a particular plant showed preferential chemotaxis toward chemical compounds found in root exudates of that plant (Bacilio-Jimenéz et al. 2003; Pedraza et al. 2010). These results suggested that chemotaxis may contribute to host-plant specificity and could largely be determined by metabolism.

Furthermore, the specificity of bacterial strains probably reflects the adaptation of the bacteria to the nutritional conditions provided by the plant and can, thus, play an important role in the establishment of *Azospirillum* in the rhizosphere of the host, as previously suggested by Reinhold et al. (1985).

6.4 Plant Growth Promotion: Mechanisms

Azospirillum species influence plant growth through versatile mechanisms; they include N_2 fixation, phytohormone production (e.g., auxins, cytokinins, and gibberellins), increased nutrient uptake, enhanced stress resistance, vitamin production, siderophores and biocontrol, and some of them do P solubilization (Steenhoudt and Vanderleyden 2000; Dobbelaere et al. 2003; Seshadri et al. 2000; Rodriguez et al. 2004). However, the PGP trait ACC (amino cyclopropene carboxylic acid) deaminase activity was absent in *A. brasilense* (Holguin and Glick 2003). Some of these features are discussed as follows:

6.4.1 Phytohormone Production

Hormones exist in nature as molecules that regulate growth, development and differentiation of cells and tissues. They act as chemical messengers and are present in small amounts. In plants we call them phytohormones and they have been, in classical terms, divided into five classes: auxins mostly indole-3-acetic acid – IAA, cytokinins (CK), giberellins (GA), abscisic acid (ABA) and ethylene (ET). However, recently new molecules are also included as regulatory compounds, such as jasmonates, brassinolides, salicylic acid, polyamines, nitrous oxide, and strigolactones that are also related to regulation of the defense reactions of plants against pathogens (Santner et al. 2009). But bacteria also produce a wide variety of these signaling molecules and influence plant growth. *Azospirillum* is a well-known bacterium that produces high amounts of auxins "in vitro" and different pathways are involved in this production. The IAA is the best characterized and the most abundant member of the auxins family (Woodward and Bartel 2005). The best characterized pathways in *Azospirillum* auxin production is via indole-3-acetamide (IAM) and indole-3-pyruvate (IPyA) intermediates, both well described by Spaepen et al. (2007).

The root morphology is altered by the inoculation of *A. brasilense*, and the knowledgement is accumulated on data based on this species as it is one of the oldest ones of the genus. Several mutants of the IAA biosynthetic genes were developed, describing the action of these mutants on the development of the root system (Dobbelaere et al. 1999). Also the environmental conditions regulate the action of the mutants and affect root colonization. For example, IAA production and expression of the key gene *ipcC* have shown to be increased under carbon limitation, during reduction in growth rate and at acidic pH values (Ona et al. 2003,

2005; Vande Broek et al. 2005). This strategy is important when plant exudates become limited, so the signaling molecule is produced by the bacteria and the root senses to continue emitting lateral roots and root hairs, which are sources of exudates to maintain the bacterial population on the roots. Unfortunately, all these measurements are performed under experimentally controlled conditions, with a single bacterial species colonizing the plant. But how these signaling molecules act in nature, with a diverse population of microorganisms competing for a single source of carbon and are influenced by the environment? It is still a gap to solve. The use of model plants such as *Arabidopsis* colonized by PGPB together with quantitative imaging of roots can contribute to a new view of this question.

Azospirillum also produced CK, a phytohormone with a broader group of molecules and derived from ^{6}N-substituted aminopurines. They are widespread among other PGPB and their spectrums do not differ from the molecules produced by plants. CKs are produced in the root tips and developing seeds, and then they are transported to the shoot where they regulate several processes such as cell division, leaf expansion, and delaying of senescence (Spaepen et al. 2009). But in the opposite site of auxins, the insights into the role with plant–microbe interaction are related only to the measurements of bacterial production on bioassays as the development of mutants is not easy to obtain. The effect is based on the balance between auxins and CKs produced by the partners.

Another class of phytohormones that *Azospirillum* also produces is the GA; these compounds are involved in division and elongation of plant cells and influence almost all stages of plant growth. Several species of the genus produce different GAs and, in addition, they metabolize exogenously applied GA. The exact mechanism of production is not described, but Lucangeli and Bottini (1996, 1997) showed that a maize line dwarf-1 (dwarfism induced by inhibitors of the GA biosynthesis) could be reverted by the inoculation with *A. brasilense* and *A. lipoferum*. The species *A. brasilense* also produces ABA, but the mechanism has not yet been proved (Cohen et al. 2008). As all hormones interact with each other, ABA can interfere with the CK pool, as it interferes with its synthesis as cited by Spaepen et al. (2009).

After all these years and with some many new species described, it will be necessary several more years to cover the complexity of *Azospirillum* phytohormone interaction with plants. Furthermore, most of these data are based on "in vitro" tests using cultivated bacteria and measuring the production in a vial. Hence, they cannot be directly correlated with plant exudates (carbon source variable), population size (lower than in vials), and influence of the environment (including biotic and abiotic factors) as it happens in natural environmental conditions.

6.4.2 Siderophore Production

Siderophores are low-molecular-mass (<1,500 Da) compounds with high iron affinity that allows soil microorganisms to sequester and solubilize ferric iron in iron-poor environments, preventing any soil-borne pathogens to proliferate due to iron

limitation (Chaiharn et al. 2009). As other soil bacteria that have to compete with microorganisms for the limited available iron, *A. lipoferum* express siderophore-mediated iron transport systems. QS seems to negatively regulate siderophore synthesis in the strain used by Boyer et al. (2008), the B518, whereas no regulation was observed in TVV3 (the other strains tested by the same authors isolated from the rice rhizosphere). In soil, where the AHL concentration is low due to the small population density, high diffusion or AHL inactivation allows B518 to produce siderophores and thus being more competitive in acquiring iron. Different *A. brasilense* strains, isolated from strawberry plants, produce siderophores (Pedraza et al. 2007), and it was found that the amount produced is related to the origin of the bacterial strain (rhizospheric or endophytic), the endophytic being the best producers.

6.4.3 P-Solubilization

Phosphorus (P) is one of the major elements in plants, but especially in tropical soils, its availability is very low (<5% of the total P pool). Microorganisms can solubilize insoluble mineral P by releasing phosphatases (organic-P) or producing organic acids (release inorganic-P). This process is common for soil bacteria (>40% of culturable ones). About the genus *Azospirillum*, this feature is not entirely known yet as many attempts to determine its P-solubilizing capacities failed due to the experimental conditions (e.g., growth culture media). However, Seshadri et al. (2000) reported in vitro inorganic P-solubilization by *A. halopraeferans*. In addition, in vitro gluconic acid formation and P-solubilization from sparingly soluble phosphorus sources by two strains of *A. brasilense* (Cd and 8-I) and one strain of *A. lipoferum* JA4 were reported by Rodriguez et al. (2004). Strains of *A. brasilense* were capable of producing gluconic acid growing in soluble calcium phosphate medium when their usual fructose carbon source was amended with glucose. At the same time, there was a reduction in pH of the medium and released of soluble phosphate. To a greater extent, gluconic acid production and pH reduction were also observed for *A. lipoferum* JA4. These data add to the very broad spectrum of PGP abilities of this genus.

6.4.4 Nitrogen Fixation

Biological nitrogen fixation (BNF) is a well-described process especially for legume in association with rhizobia. As they possess a nodule that can be counted and plants without nodule or nitrogen fertilizer are complete nitrogen deficient, it was easy to propose the mode of action, describe the amount produced by BNF, and understand the several steps of colonization and fixation.

For other plants that do not possess nodules to fix nitrogen, the contribution is not 100% of the total N accumulated in tissues. The best known results of grasses

are related to sugarcane where it was proved that in controlled condition, 70% of the nitrogen accumulated came from the biological process (Urquiaga et al. 1992) and in field assays it was reduced to 30% (Yoneyama et al. 1997). Then another problem arises, who is the responsible for the BNF observed? As we already know, only 2–3% of the total bacteria are described, and every year new genus and lots of species are described. It is not easy to follow the same for the ecology of a new strain, plant localization, and experiments performed in order to calculate the impact of a single species in the BNF process. So we are describing bacteria without knowing its significance to plant nutrition.

A lot of field assays were performed in order to evaluate the effect of its inoculation on several grasses, and some authors have described them in detail. Okon and Labandera-González (1994) concluded that a significant increase in yields from 5 to 30% could be achieved by the inoculation with *Azospirillum*, especially with lower doses of nitrogen fertilizers. Also the effect was attributed to phytohormone production rather than nitrogen fixation. Fages (1994) showed that the success of using *Azospirillum* is around 50–75%, which is more than the normal measure expected for leguminous inoculation that is around 50%. Sumner (1990) described positive results to *Azospirillum* inoculation and most of the experiments on wheat were performed between 1983 and 1985. Recently, rice cropped under rain-fed conditions in Tucumán, AR, showed clear effects of *Azospirillum* inoculation when compared to N-fertilized noninoculated controls (Pedraza et al. 2009).

6.5 Agricultural Application

The utilization of bacteria as an inoculant product is the ideal goal based on the performance of rhizobial inoculants, especially in Brazil, where 100% of the soybean production use the bacteria and not the fertilizers to obtain 100% of the N necessary for the plant nutrition. After so many years of testing, isolating and describing *Azospirillum*, some efforts were also carried out in relation to have a commercial product using this bacterium. The technology is also based on the rhizobial product that is applied on the seed cover in a mixture with peat or using different kinds of liquid formulations.

At the beginning, only *A. brasilense* was the elected one as inoculant. In the United States, a product called Azo-Green™, produced by a company called Genesis Turfs Forages, was recommended to be applied on the seeds to improve germination, root system, drought resistance, and plant health. In Italy, Germany, and Belgium another product containing a mixture of *A. brasilense* (strain Cd) and *A. lipoferum* (strain Br17) was formulated in a mixture of vermiculite or in liquid formulation. The commercial name was Zea-Nit™ and was produced by Heligenetics and they recommended the reduction of 30–40% of the N fertilization of the plants. In France another AzoGreen™ was used in a maize application in the Agbasar Station, at Northeast of Tongo, Africa, with the increment of 100% in yield using a strain isolated by Fages and Mulard (1988), the CRT-1. In Mexico,

a product called "Fertilizer for Maize" was developed by the University of Puebla and applied in 5,000 ha in 1993 (Okon and Labareda-González 1994). More recently, in 2008, another inoculant product based on *Azospirillum* was developed to coffee plants in Mexico, and its application showed reduction of time during the three phenological plant cycle (Jímenez Salgado – http://www.proyectocinco.com/notas/reportaje04abr08.htm). Uruguay also had a product called Graminante™ that was commercialized as a powder mixed with calcium carbonate.

But regarding these bacteria, which are different for each country, why are they the best ones? Perrig et al. (2007) evaluated phytohormone and polyamine biosynthesis, siderophore production, and phosphate solubilization in two strains (Cd and Az39) of *A. brasilense* used for inoculant formulation in Argentina during the last 20 years. Siderophore production and phosphate solubilization were evaluated in a chemically defined medium, with negative results. Phytohormones IAA, cytokinins called zeatin, GA3, ABA, ethylene, and growth regulators putrescine, spermine, spermidine, and cadaverine (CAD) were found in culture supernatants of both strains. IAA, zeatin, ethylene, and polyamine CAD were found in all two strains. Az39 and Cd showed differential capability to produce the five major phytohormones and CAD in chemically defined medium. This fact has important technological implications for inoculant formulation as different concentrations of growth regulators are produced by different strains or culture conditions.

It is also important to maintain the good quality of the inoculant in order to provide efficient root colonization or invasion. It is necessary to adjust the cell density (10^9 minimum per gram) shelf life, free of contaminants and agronomically proved that the strain applied is capable to maintain the yield in the absence of a reduced dose of nitrogen or improve crop yield, in the presence of nitrogen application (PGPB action). In 2009, a company in Brazil began to sell a product based on *Azospirillum* strains for maize and rice application. In Argentina, there are several companies producing and selling inoculants based on *A. brasilense* that are being applied as solid (powder) or liquid formulations in different commercial crops (e.g., rice, maize, wheat, sunflower, sorghum, etc.).

Presently, with the reality of having to produce more food at a lower cost, without environmental pollution, biological fertilization with PGPB is an alternative for a sustainable agriculture. Although beneficial effects of inoculating with *Azospirillum* sp. has been widely described, the efforts in isolating new strains and assessing their PGP characteristics in natural environmental situations must continue in order to support their agricultural use as inoculant or biofertilizer.

6.6 Final Considerations

From the numerous scientific literature available today, it is clear that the *Azospirillum*–plant interaction is a natural and ubiquitous phenomenon. With the increasing understanding and knowledge of the benefits conferred to the host by this

association, we are one step forward to developing and improving an environment-friendly nutrient source and biocontrol agent for agronomically important crops. But for this, the efforts should be focused not only on describing new species, sometimes based on few bacterial isolates, but also on assessing their PGPB capacities, including their exploitation within the interspecies biodiversity.

Despite the recent advances in its biotechnological use as inoculants or biofertilizers, commercialization of this technology based on *Azospirillum* still demands extensive optimization and comprehensive study of the effects of the application. However, the prospects of this technology are promising if we take into consideration the rising cost and declining reserves of fossil fuels in the world, as well as pollution problems. In this scenario, the progressive installation of this biotechnology would mitigate environmental concerns arising from the use of nitrogenous fertilizers and its costs of acquisition, especially in developing countries, in order to support a sustainable agriculture.

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