

Azospirillum sp. as a Challenge for Agriculture

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Abstract Several processes mediated by soil microorganisms play an important role in nutrient cycling. One such process is biological nitrogen fixation (BNF) by representatives of various bacterial phylogenetic groups, which are called diazotrophs. Most studies of the *Azospirillum*-plant association have been conducted on cereals and grasses. Currently, 17 species of *Azospirillum* have been described. However, a great diversity of these bacteria continues to be revealed, and little is known of the potential applications of the many species that have been described. The *Azospirillum*-plant association begins with the adsorption and adherence

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process of these bacteria in roots. Involved in these processes is the recognition of bacterial polysaccharides by the host plant, a step that is necessary in successfully forming a positive relationship between roots and *Azospirillum*. The presence of *Azospirillum* in the rhizosphere can minimize the susceptibility to diseases caused by plant pathogens. Furthermore, the ability to produce phytohormones, mainly auxins (indole-3-acetic acid) and other molecules from secondary metabolism has been suggested to underlie the growth response to inoculation by *Azospirillum* species. These positive aspects of *Azospirillum* colonization in the roots are also responsible for the alleviation of plant stress. For all of the above-mentioned reasons, *Azospirillum* are also widely used as commercial inoculants, resulting in a significant economic impact in crop yields in many countries. In fact, solid and liquid formulations containing *Azospirillum* are marketed in various countries, such as Brazil, Argentina, Mexico, Italy, France, Belgium, Africa, Germany, Pakistan, Uruguay, India and the USA. In addition, new formulations containing *Azospirillum*, such as polymeric inoculants (alginate, agar, chitosan and gum), are already used for the improvement of many crops. This chapter summarizes the positive effects of *Azospirillum*-plant interactions and their biological importance for the improvement of agriculture worldwide.

Keywords *Azospirillum* • Biological nitrogen fixation • Biocontrol • Phytohormones • IAA

1 Introduction

Brazil has a long tradition of research with nitrogen-fixing species, and the attention to nitrogen-fixing *Azospirillum* species increased after their rediscovery by Dobereiner and Dias in the year 1976. *Azospirillum* is one of the best-studied plant growth-promoting rhizobacteria (PGPR) that are normally associated with grasses, rice, wheat and sugarcane (Bashan and De-Bashan 2010; Babalola and Glick 2012; Duca et al. 2014; Glick 2014). Presently, 17 species of *Azospirillum* have been described (in order of discovery): *A. lipoferum*, *A. brasilense*, *A. amazonense*, *A. halopraeferens*, *A. irakense*, *A. largimobile*, *A. doebereineriae*, *A. oryzae*, *A. melinis*, *A. canadense*, *A. zaeae*, *A. rugosum*, *A. picis*, *A. thiophilum*, *A. formosense*, *A. fermentarium*, *A. humicireducens* and *A. himalayense* (<http://www.bacterio.net/azospirillum.html>). Of these, *A. brasilense* and *A. lipoferum* are the most studied and well described.

Azospirillum strains are marketed in various countries, such as Brazil, Argentina, Mexico, Italy, France, Australia, Pakistan, Germany, the USA, Africa, Belgium, India and Uruguay (Hungria et al. 2010; Reis et al. 2011; Mehnaz 2015), mainly as microbial formulations with other microorganisms. The major visual effects of inoculation with *Azospirillum* are changes in root morphology that results in an increase in root elongation, the number of lateral and adventitious roots and the lengthening and branching of root hairs (Bashan and Levanony 1985;

Okon 1985; Baldani and Döbereiner 1986; Okon et al. 1988, 1991; Okon and Labandera-Gonzalez 1994; Cohen et al. 2015). These effects on root morphology permit the roots to take up more water and mineral nutrients, which leads to an increase in plant growth (Fibach-Paldi et al. 2012). Moreover, *Azospirillum* might help plants survive under stressful situations due to the induction of changes in cell wall elasticity, osmotic adjustments and the release of beneficial substances (Richardson et al. 2009; Groppa et al. 2012).

Several PGPR inoculants currently commercialized seem to promote growth through at least one mechanism: suppression of plant disease (bioprotectants), improvement of nutrient acquisition (biofertilizers), or phytohormone production (biostimulants) (Tenuta 2003; Mitter et al. 2013). Understanding the interaction between the consortium of microbial inoculants and plant systems will enable growers to harness more benefits from microbial inoculants for improving plant growth and yield (Raja et al. 2006; Sivasakthivelan and Saranraj 2013). The use of microorganisms with the aim of improving nutrient availability for plants is an important practice and proved necessary for agriculture (Babalola 2010; Figueiredo et al. 2010; Araujo et al. 2011; Rodrigues et al. 2013a, b; Bashan et al. 2014).

2 *Azospirillum*: Involvement of Polysaccharides in Attachment

Azospirillum sp. is a Gram-negative diazotrophic rhizobacteria associated with plant roots (mainly grasses). Species of *Azospirillum* exhibit chemotaxis toward a variety of root exudates like amino acids, organic acids, sugars and aromatic compounds (Okon et al. 1980; Rodriguez-Navarro et al. 2007). Chemotaxis is a widespread function in motile soil bacteria, because it affords cells with the ability to sense and navigate toward the most favorable niches for growth and represents an important attribute for plant-microbe association (Carreño-López et al. 2009; Alexandre 2010). The chemotactic response of *Azospirillum* species toward root exudates represents the first stage in *Azospirillum* colonization of root plants and is followed by attachment (Schelud'ko et al. 2009; Wisniewski-Dyé et al. 2013). Although attachment is already known, the precise mechanism that rules the attachment process remains unexplained due to its great complexity (Jofré et al. 2009; Richardson et al. 2009).

The attachment of *Azospirillum* sp. to plant roots is necessary for the formation of an active association and seems to occur in two distinct and consecutive phases (Belyakov et al. 2012; Fibach-Paldi et al. 2012); (i) adsorption and (ii) anchoring phases. In the adsorption phase, a weak binding occurs between the bacteria and the root cells mediated by the polar flagellum (Rodriguez-Navarro et al. 2007). *Azospirillum* produces one longer polar flagellum and several shorter peritrichous flagella (Fibach-Paldi et al. 2012). The *Azospirillum* polar flagellum is an important component of cell motility and is divided into a basal body, hook and filament (Lerner et al. 2010; Belyakov et al. 2012). The filament is composed of numerous

identical flagellin molecules, a protein with the C- and N-terminal conserved and a variable middle region (Belyakov et al. 2012). The variability of middle region of the flagellin is related to their antigenic and adhesive properties and the flagellin glycosylation mediates the symbiosis with eukaryotic organisms (Iwashkiw et al. 2013; Merino and Tomás 2014). In cooperation with the flagellin protein, capsular polysaccharides (CPS) are also involved in the adsorption phase of attachment of the *Azospirillum* to the root surface (Lerner et al. 2010). It is reported that *Azospirillum* secretes CPS, and this polysaccharide seems to mediate the adhesion of bacteria to surfaces (Dutta and Podile 2010). CPS is a type of external polysaccharide bound to the outer membrane by a covalent bond (Wisniewski-Dyé et al. 2013). In the CPS of the *A. brasilense* Sp7, a glycosylated lectin with a molecular mass of 36 kDa was identified with specificity to L-fucose and D-galactose (Sigida et al. 2013). Lectins are sugar-binding proteins that can specifically and reversibly recognize and bind to carbohydrates present on the plant root surface. An outer membrane lectin of 67-kDa and produced by *A. brasilense* Sp7 could be involved in adhesion processes (Mora et al. 2008).

Azospirillum strains produce various lectin types, and are possibly involved in *Azospirillum* cell adhesion to the root surface (Aleñkina et al. 2014a). The diversity and complexity of these lectins, probably due to the high pleiotropic capacity of *Azospirillum*, ensures their adaptation to different host plants (Mora et al. 2008). Lectins produced by *A. brasilense* induce several signaling systems in wheat roots as part of the recognition in the initial stages of development of plant-bacteria association following the ligand-receptor interaction principle (Fig. 1). However, specific receptors present in the *Azospirillum* cell surface can bind to

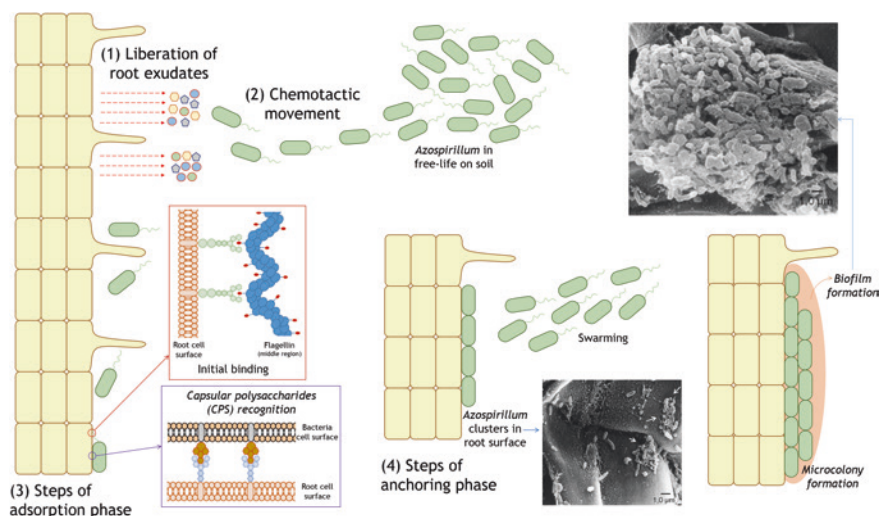


Fig. 1 The steps of attachment of *Azospirillum* sp. to plant roots (the scanning electron micrographs (SEM) are roots colonized by *A. brasilense* and arrows indicate zones with granular-like material; SEM from Guerrero-Molina et al. (2012))

the lectins present in the plant root surface (Scheludko et al. 2009). The binding of lectin WGA (wheat germ agglutinin) to cell receptors of *A. brasilense* Sp245 alters bacterial cell metabolism and acts as a signal molecule in the *Azospirillum*-plant association (Aleñkina et al. 2014b).

The second phase of attachment of *Azospirillum* sp. to the plant root surface is mediated by exopolysaccharides (EPS), a polysaccharide weakly associated with the outer cell membrane or totally released into the extracellular medium (Wisniewski-Dyé et al. 2013). In the anchoring phase, the second stage of the attachment process, the *Azospirillum* becomes irreversible and firmly attached to the root surface and other bacteria that are also entrapped, forming clusters at the attachment site (Winik et al. 2009; Guerrero-Molina et al. 2012). The EPS mediates anchoring due to their involvement in the cell-to-cell aggregation phenomenon and its special interaction with bacterial envelope components (Mora et al. 2008). The EPS composition seems to be an important determinant for aggregation ability in *Azospirillum* strains (Fibach-Paldi et al. 2012). This observation is sustained by the fact that in the aggregation phase, the dominant sugar in the EPS structure of *A. brasilense* is L-arabinose (Bahat-Samet et al. 2004; Mora et al. 2008).

Together with CPS and EPS, the lipopolysaccharides (LPS) of *Azospirillum* contribute to the bacteria-plant association due to their responsibility in the immunospecificity of the bacterial cell and thus are involved in direct interactions with plants (Molinaro et al. 2009; Fedonenko et al. 2013; Sigida et al. 2013). LPS is a glycoconjugate present in the cell-surface of *Azospirillum* strains and exhibits a lipid moiety (lipid A), which anchors the molecule in the membrane, and a chain of oligosaccharides covalently linked to lipid A (Sigida et al. 2013; Shelud'ko et al. 2014). The polysaccharide portion includes a central oligosaccharide (core) and an *O*-polysaccharide moiety (OPS) (Fedonenko et al. 2011). The CPS and LPS show marked structural differences, and this is related to the involvement of these structures in different stages of the *Azospirillum*-plant association (Smol'kina et al. 2010; Fedonenko et al. 2011; Shelud'ko et al. 2014).

The OPS structure present in *Azospirillum* LPS is responsible for serological cross-reactions and the basis for the classification into a certain serogroup (Fedonenko et al. 2013). *Azospirillum* strains are divided into three serogroups (Shelud'ko et al. 2014). *Azospirillum* from serogroup I possess a linear homopolymeric D-rhamnan OPS (Boyko et al. 2012), while a heteropolysaccharide OPS occurs in serogroup II (Konnova et al. 2008) that is precipitated with LPS antibodies of *A. brasilense* Sp7 (Sigida et al. 2014). In serogroup III, OPS is composed of a main chain with an oligosaccharide motif formed by three L-rhamnose residues linked with a side chain formed by a D-glucose homopolysaccharide (Fedonenko et al. 2011). The differences in OPS structures are related with host recognition (Fedonenko et al. 2013). In fact, *Azospirillum* strains of serogroup I are usually encountered in association with wheat, while strains of serogroup II and III have an association with other gramineous plant (Sigida et al. 2014).

Azospirillum cells attached to plant roots exhibit a rounded and swollen format, similar to a cyst, and are metabolically active in the rhizosphere (Hou et al. 2014).

After *Azospirillum* cells attachment, the swarming occurs, a process by which a bacterial group rapidly advances on surfaces until specific sites and colonize in a coordinated manner (Verstraeten et al. 2008). In fact, swarming enables the rapid colonization of host tissues and formation of microcolonies which occurs simultaneously with biofilm formation (Bogino et al. 2013). Biofilm is a bacterial aggregate associated with a surface, typically enclosed in an extracellular matrix, and probably composed of microbial EPS (Kadouri et al. 2003; Monds and O'Toole 2009). *Azospirillum brasilense* is reported as a biofilm producer (Guerrero-Molina et al. 2012), and this feature helps *Azospirillum* cells anchor and colonize the root surface (Winik et al. 2009). Overall, swarming and biofilm formation across the roots is important for long-term colonization.

3 *Azospirillum* in the Biological Control of Pathogens

Azospirillum brasilense have been suggested as plant-growth promoting bacteria (Bashan and Holguin 1998). The bacteria of the genus *Azospirillum* inhabit the plant's rhizosphere and sometimes develop an endophytic relationship with the host plant. However, in most cases, their relationship is associative, with a partial supply of nitrogen fixed by the microbial process, known as atmospheric nitrogen biological fixation, and a chemical reaction catalyzed by a dehydrogenase enzyme (Hungria et al. 2010). The success of the relationship also depends on the ability of the bacteria to colonize the host plant rhizosphere, although no consistent results demonstrate specificity in the bacteria-host plant relationship.

Even though the genus *Azospirillum* is not considered directly related to biological control, some reports in the literature show results of moderate biological control in some diseases, such as galls caused by *Agrobacterium tumefaciens* and leaf and vascular diseases caused by bacteria. In addition, research has also shown the genus to inhibit the growth of non-pathogenic microorganisms in the rhizosphere of plants (Somers et al. 2005). The mechanism of action responsible for the benefits found in the growth of plants has also been studied in every bacterial genus of interest, which discusses, among other things, the participation of different microbial molecules involved in such mechanisms (Kloepper et al. 2004). The presence of *A. brasilense* can change plant physiology, especially the production of several phytohormones, such as auxin, gibberellin, cytokinin and ethylene (Dobbelaere et al. 1999).

Some chemical compounds produced by bacteria (when in contact with the plants), such as those of the genus *Azospirillum*, can interfere with plant metabolism and the active frequency of the plant defense. The plant defense system is controlled by different metabolic pathways that are triggered by various chemical factors associated with the presence of chemical and biological inducers (Kuc 1983). The bacterial effect, when present in the plants rhizosphere, can be observed in two specific routes of metabolic defense in plants. These routes include jasmonic and salicylic acid, of which the latter is most often associated

with chemical inducer agents or pathogens (Romeiro 2000). With regard to bacteria that promote plant growth, the induced systemic resistance promotion (ISR) is more associated within their mechanism of action, which reinforces the positive effects of these bacteria in promoting growth or biological control of pathogens (Mariano and Kloepper 2000). The ISR, when involved in the action of non-pathogenic microorganisms in the rhizosphere, does not involve the salicylic acid signaling pathway or induction of proteins related to pathogenesis because it is activated in this resistance-signaling pathway of jasmonic acid and ethylene (Hoffland et al. 1995; Pieterse et al. 1998). When plant beneficial bacteria colonize the root system, the constituent molecules of the bacterial cell, or those synthesized by it, elicit a biochemical signal. This signal is translocated to distant sites, which activates genes that code for the synthesis of dynamic resistance components and thus induces the expression of a systemic resistance (Romeiro 2000).

Romero et al. (2003) demonstrated that the activity of phytohormones in two strains of *Azospirillum* sp. promoted growth in tomatoes and modified the plant susceptibility to bacterial diseases. The success of this interaction depends on the plant genotype as well as the pathogen characteristics. In a review of root diseases in cassava, it was determined that the inoculation of *A. brasilense* reduced the incidence rates of disease/infection compared to the non-inoculated controls. This species can restrict the proliferation of other non-pathogenic bacteria in the rhizosphere and bacteria, such as *Pseudomonas syringae* pv. *tomato* (Bashan and De-Bashan 2002), which probably occurs due to competition or the resistance induction phenomenon in the host. Biological control reports provided by *A. brasilense* are related to growth inhibition of *Agrobacterium tumefaciens* and phytopathogenic fungi. This inhibitory activity may be related to compounds detected in the supernatant of *Azospirillum* sp. during growth (Fig. 2); however, the mode of action is not well defined (Somers et al. 2005). According to these authors, the

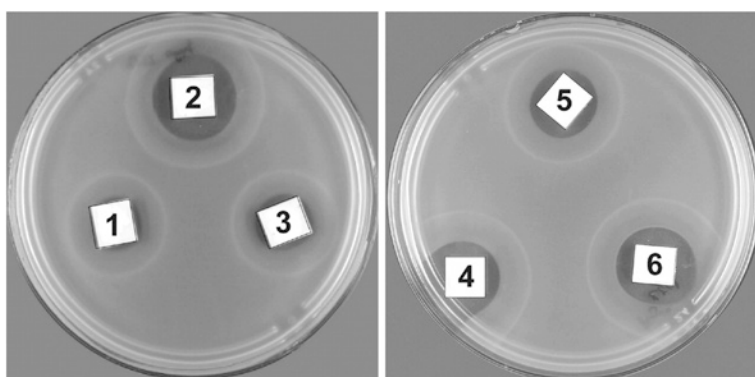


Fig. 2 Determination of the antimicrobial activity of supernatant extracts (10 μ L) isolated from *A. brasilense* Sp245 cultures grown in MMAB supplemented with 0.5 mM tyrosine, (1) 0.5 mM phenylalanine, (2) 0.5 mM tryptophan, (3) 0.5 mM phenylpyruvate, (4) 0.5 mM prephenate, (5) or 0.5 mM chorismate (6) by the paper disk method (Somers et al. 2005)

antimicrobial compound produced by *Azospirillum* sp. was identified as phenyl acetic acid of the auxins group. This molecule has been used as a defense mechanism in bacterial competition in its ecological niche. The presence of hormones can alter both plant growth and influence the metabolism and survival of microorganisms in the environment.

Microbial iron chelators known as siderophores are produced by *A. brasilense* and showed antifungal activity in vitro against *Colletotrichum acutatum* M11 strain. Siderophores are molecules that sequester iron from the environment; thus, this essential metal for microbial growth can be crucial in causing deficiency in the growth of certain microbial species. A reduction of anthracnose symptoms in strawberries previously inoculated with *A. brasilense* was also observed. These results suggest the use of bacteria for disease control strategies in the strawberry tree (Tortora et al. 2011). Some chemical compounds (not yet identified) produced by *A. brasilense* sp245 promoted, under controlled conditions, the reduction of mycelial growth of *Rhizoctonia solani* (Russo et al. 2008; Vettori et al. 2010). In another study, chemical inhibitors of fungal growth were also characterized as volatile, and after extracting the growth supernatant of *A. brasilense*, these substances reduced the growth of *Fusarium graminearum* (after addition to the fungal growth medium culture) (Abdulkareem et al. 2014).

With regard to the benefits provided by *Azospirillum* sp. for the biological control of diseases, at this stage, it is still not clear which mechanism of action is critical for the success of this activity. The production of hormones, siderophores and the ability to fix atmospheric nitrogen guarantee benefits and increased competence of *A. brasilense* in colonization and its ability to remain in the rhizosphere of plants. This can promote plant growth and reduce the presence of pathogenic microorganisms in the surrounding soil. Furthermore, the presence of these bacteria in the rhizosphere confers several benefits for plant growth and nutrition, which indirectly increases the resilience capacity.

4 Mechanisms by Which *Azospirillum* Affects Plant Growth: Hormones and Metabolites

Plant-microbe interactions are affected by many different regulatory signals, and the root exudates stand out among them (Spaepen et al. 2009). The root exudates play a key role in the plant-microbe interaction, stimulating the bacterial chemotaxis and mediating the root colonization and the selection of microorganisms driven by the host (Mitter et al. 2013). Therefore, root exudates play an important role in developing microbial communities in the different compartments of plants (Fibach-Paldi et al. 2012). As compensation to root exudates secreted by plants, the microorganism plant association may improve plant growth and health by synthesis of vitamins, antibiotics, enzymes and phytohormones (Cohen et al. 2015). Phytohormones are organic substances that at a very low concentration stimulate a physiological response. Currently, in addition to the five classic plant hormones

(auxins, cytokinins, gibberellins, abscisic acid and ethylene), other phytohormones have been identified, such as jasmonate, brassinosteroid, nitric oxide and strigolactone (Shan et al. 2012).

Azospirillum strains have been reported to increase plant growth through the action of carbohydrates, polyamines, amino acids, peptides, lectins and enzymes that are released in the extracellular medium (Cassán et al. 2009a; Richardson et al. 2009; Bashan and De-Bashan 2010). However, many authors report that *Azospirillum* species are able to enhance plant growth due to the self-production of hormone and by inducing synthesis of these compounds in the plant tissues (Chamam et al. 2013; Duca et al. 2014; Cohen et al. 2015). In general, the phytohormones works in complex networks that include responses in cross talk and feedback, and therefore, it is difficult to establish the specific role of a given hormone in the plant response (Glick 2014). *Azospirillum* species are able to produce and secrete phytohormones, mainly auxins, gibberellins (GAs), cytokinins (CK) and nitric oxide, which act as signals and effectors for plant growth promotion (Spaepen et al. 2008; Bashan and De-Bashan 2010; Couillerot et al. 2013; Duca et al. 2014).

Several studies have reported the presence of auxins in the supernatant of *Azospirillum* cultures (Cassán et al. 2014; and references therein). Quantitatively, indole-3-acetic acid (IAA) seems to be the most important auxin produced by *Azospirillum* (Glick 2014; Mehnaz 2015); however, some reports suggest that indole-3-butyric acid (IBA) is also largely produced (Couillerot et al. 2013). In accordance with Duca et al. (2014), IBA probably serves as an important source and reserve of IAA in *Azospirillum* strains. *Azospirillum* sp. produce IAA during all growth stages (Malhotra and Srivastava 2009) and four pathways exist for IAA biosynthesis (Duca et al. 2014): three tryptophan-dependent pathways [indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM) and tryptamine (TAM) pathways] and one tryptophan-independent pathway (Fig. 3). The IPA pathway is of major significance in *Azospirillum* and provides 90 % of the IAA synthesized (Glick 2014).

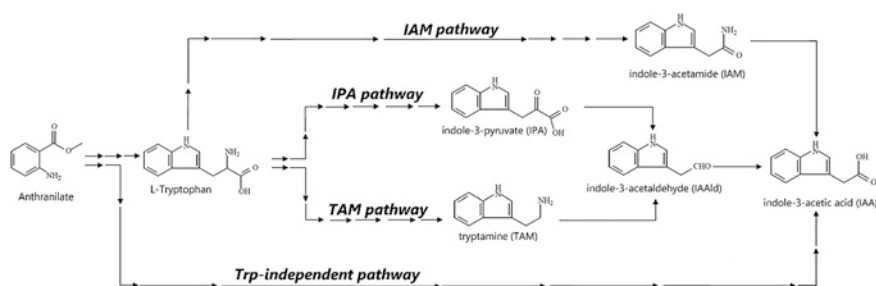


Fig. 3 Indole-3-acetic acid (IAA) pathways identified in *Azospirillum* species: the indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA) and tryptamine (TAM) pathway (tryptophan-dependent pathways) and a tryptophan-independent pathway starting with anthranilate (Cassán et al. 2014; Duca et al. 2014)

In the plant-*Azospirillum* interaction, IAA can also be a reciprocal signaling molecule that maintains the symbiotic relationship (Malhotra and Srivastava 2009) and has probably been shaped by co-evolutionary processes between the bacteria and their host plant (Berg 2009; Walker et al. 2011, 2012; Drogue et al. 2012). In fact, some evidence indicates that phytohormone synthesis in *A. brasilense* is strain-specific (Di Salvo et al. 2014). These authors studied the Sp7, Cd, Az39, 40 and 42 strains of *A. brasilense* and reported that the 42M strain displayed higher levels of IAA production than the other strains. Many authors have reported the positive effects of auxin synthesized by *Azospirillum* in plants (Malhotra and Srivastava 2009; Glick 2014 and references therein). IAA regulates the plant cell cycle, tropism, apical dominance and senescence (Mehnaz 2015). In addition, under environmental fluctuations and nutrient limitations, specifically nitrogen, carbon and phosphorus, the IAA levels are increased (Malhotra and Srivastava 2009).

IAA produced by *A. brasilense* alters the root morphology and proliferation in wheat seedlings (Dobbelaere et al. 2001), and is responsible for increases in root and shoot systems. In fact, the inoculation of sugarcane with *Azospirillum* sp. results in significant increases in root dry weight (upper 70 %; Moutia et al. 2010), and wheat inoculated with *A. brasilense* exhibit increases in shoot growth (Spaepen et al. 2008). In addition, IAA affects photosynthesis, the biosynthesis of metabolites and other phytohormones, such as CK and GAs (Ilyas and Bano 2010; Mehnaz 2015). Tien et al. (1979) documented the first report of CK production in *A. brasilense* in 1979; however, little is currently known about the CK produced by *Azospirillum* (Cássan et al. 2014). In plants, CK regulate cell division, and have been associated with shoot and root morphogenesis (Spaepen et al. 2009). Zeatin, the major CK type, has been reported in *A. brasilense* and *A. lipoferum* (Molina-Favero et al. 2007; Esquivel-Cote et al. 2010).

GAs are one class of phytohormones produced and secreted by *Azospirillum* (Mehnaz 2015). GAs are complex compounds composed of terpenes that share a common GA ring (Yamaguchi 2008). Currently, several GA types have been identified; of these, GA₁, GA₃, GA₄ and GA₇ are the types that show functions of phytohormones, and therefore, regulate different aspects of plant growth (Cassán et al. 2014). *Azospirillum* species, specifically *A. brasilense* and *A. lipoferum*, are known to produce GA₁ and GA₃ and GA₃ is the major type of GA identified (Bottini et al. 1989; Jansen et al. 1992; Piccoli and Bottini 1996; Lucangeli and Bottini 1997; Ilyas and Bano 2010). Manivannan and Tholkappian (2013) recorded a production of GA up to 3.3 µg (per 25 mL⁻¹ broth) in 20 different *Azospirillum* strains isolated from the tomato rhizosphere.

Although it has been known for a long time that *Azospirillum* synthesizes and metabolizes GAs (Bottini et al. 1989), their mechanism of production is poorly known (Mehnaz 2015). Lucangeli and Bottini (1997) were the first to describe the capacity of *Azospirillum* sp. that produce GAs in plants. GAs promote cell division and the elongation of primary roots, and play an important role in lateral root development (Bottini et al. 2004). In maize, GAs promote shoot elongation and growth and increase root hair abundance (Fulchieri et al. 1993). In rice inoculated with *A. lipoferum*, GA improves nitrogen uptake and increases the dry

mass, height and yield (Bottini et al. 2004). In maize plants treated with an inhibitor of GA biosynthesis, results show that GAs produced by *Azospirillum* positively affects plant growth (Lucangeli and Bottini 1997). A study in maize treated with prohexadione (GA biosynthesis inhibitor) by foliar spraying and inoculated with *A. lipoferum* reported an increase in root growth (Cohen et al. 2009).

Data from studies with GA-deficient plants with the dwarf phenotype show that GAs of *A. brasilense* and *A. lipoferum* were responsible for reversal of dwarfism in maize and rice (Cassán et al. 2001). Another important role of GA is the interruption of dormancy during seed germination because its hydrolytic enzymes, α -amylase and protease, are induced in seeds of grasses and cereals, and this facilitates endosperm mobilization (Mehnaz 2015). The seeds of soybean and wheat exhibit an increase in germination when treated with *A. brasilense*, and this response seems related to the high GA production of *A. brasilense* (Bacilio et al. 2003; Cassán et al. 2009b). Furthermore, environmental factors can modify the GA production by *Azospirillum* (Cassán et al. 2014). In this sense, Piccoli et al. (1999) showed that the availability of O₂ and the osmotic potential reduces the GA₃ production in *A. lipoferum* (~50 %), and this response was considered a compensatory mechanism that seems to be activated in water stressed situations.

GAs and abscisic acid (ABA), produced by *Azospirillum* strains, seem to contribute to water stress alleviation in plants (Cohen et al. 2009, 2015) and in plant defense mechanisms (Vacheron et al. 2013). ABA is a phytohormone induced in response to environmental stress, such as water or salt stress (Bauer et al. 2013). The inoculation of maize plants with *A. lipoferum* enhances ABA levels and plant tolerance to drought (Cohen et al. 2009). Likewise, the synthesis of ABA by *Azospirillum* species increases when sodium chloride (commonly used to mimic salt stress) is added to the culture medium (Cohen et al. 2008; Dodd et al. 2010; Cohen et al. 2015). Ilyas and Bano (2010) report that *Azospirillum* strains isolated from water stressed conditions exhibited higher production of ABA. Moreover, *Arabidopsis* seedlings doubled ABA levels when inoculated with *A. brasilense* sp245 (Cohen et al. 2008). These results reinforce idea of a protective role for ABA synthesized by *Azospirillum* (Fig. 4).

Plant tolerance to environmental stress mediated by *Azospirillum* may involve ABA or compatible solutes like proline, polyamines and trehalose (Richardson et al. 2009; Cohen et al. 2015). *Azospirillum* sp. are known to produce polyamines and amino acids in culture media (Cassán et al. 2009a; Bashan and De-Bashan 2010). Spermine, spermidine, putrescine and cadaverine are organic polymers generically named polyamines and are related to root growth and stress mitigation in plants (Gupta et al. 2013). In rice seedlings inoculated with *A. brasilense*, at least in part, cadaverine production induces root growth and mitigates osmotic stress in rice (Cassán et al. 2009a). Maize plants treated with *A. brasilense* modified to over-produce trehalose were more resistant to drought and improved biomass production more than plants treated with a wild type of *A. brasilense* (Rodríguez-Salazar et al. 2009). In addition, in their hosts, *Azospirillum* sp. induce the biosynthesis of phenylacetic acid, bacteriocins and siderophores, which are secondary metabolites with antimicrobial activity (Walker et al. 2011; Vacheron et al. 2013).

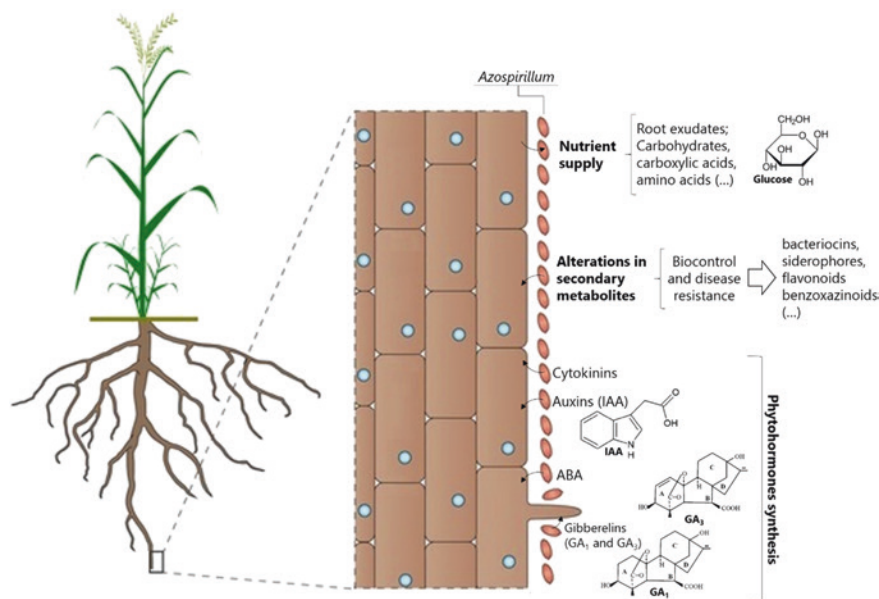


Fig. 4 The positive effects of *Azospirillum* in plant roots. The plants provide nutrients while *Azospirillum* can promote phytohormone synthesis and positive alterations in secondary metabolites. IAA Indole-3-acetic acid. ABA Absciscic acid

The inoculation of *Azospirillum* modifies the content of secondary metabolites in the host plant, and in accordance with Walker et al. (2011), these alterations are more intense than that in primary metabolism. Alterations in the secondary metabolite profile were reported in rice plants in response to *Azospirillum* inoculation, and the major metabolites affected were flavonoids and hydroxycinnamic derivatives (Chamam et al. 2013). In maize inoculated with *Azospirillum*, variations in benzoxazinoids were registered, which are molecules related to plant resistance against pathogens in both roots and shoots (Walker et al. 2012). These molecules are elicited in the host plants when inoculated with *Azospirillum* and are responsible for disease resistance. Overall, considering the beneficial interactions between plants and *Azospirillum*, their use as an inoculant represents an environmentally friendly strategy for agriculture worldwide.

5 *Azospirillum*: Inoculants and New Products

Plant biostimulants include both substances and microorganisms that enhance plant growth, and the global market is estimated at over 2 million dollars by 2018; however, the definition is still evolving, mostly due to its large scope. Both the American and the European definitions include several potential effects, but

specify that these products may not establish nutritional guarantees, although they may increase fertilizer efficiency (Calvo et al. 2014). This broad class of products is especially important for agricultural systems in which external inputs are limited, due to philosophical, ecological, or economic reasons (Bhardwaj et al. 2014).

Bacteria-based inoculants used as plant biostimulants are usually classified as PGPR and include several different mechanisms ranging from BNF to plant protection from pathogens (Maheshwari 2010; Bhattacharyya and Jha 2012; Ahemad and Kibret 2014; Nadeem et al. 2014). One of the major groups of bacteria-based inoculants is collectively known as biofertilizers, and these bacteria improve the nutrient status of the crops through BNF, increasing nutrient availability and/or root growth promotion, and they usually work through a combination of benefits that are hard to experimentally differentiate (Perez-Montano et al. 2014). One of the most widely studied genera of biofertilizers is *Azospirillum* (Laslo et al. 2012; Almaghrabi et al. 2013; Reddy and Saravanan 2013; Nadeem et al. 2014; Perez-Montano et al. 2014).

Inoculants based on *Azospirillum* have already been commercialized in Mexico, France, India and Brazil (Hungria et al. 2010; Sivasakthivelan and Saranraj 2013; Trujillo-Roldán et al. 2013) and have been promoted and studied since at least the 1970s, and thorough review published in 1994 (Okon and Labandera-Gonzalez 1994). The authors indicated a 60–70 % success rate using *Azospirillum* inoculants with a typical gain of 5–30 % for several of the most important cereal crops. However, wide variations in their effects are still routinely found in field experiments (Hungria et al. 2010; Lana et al. 2012; Turan et al. 2012; Castañeda-Saucedo et al. 2013; Romero et al. 2014).

These variations happen due to a wide range of reasons, from inoculant production through storage, field use and the several ecological and environmental effects known to affect the results. A major point that should be considered by both inoculant producers and researchers is that the end-user is mostly interested in the product effect on the crop, not on the bacteria being used to achieve that end, its physiology or ecology. Because these effects largely depend on the strain used and its population on the product when applied, these aspects should probably be the main research focus in this field (Sivasakthivelan and Saranraj 2013). Strain selection should be based on a multitier approach, similar to that conducted with rhizobial legumes or the crop breeding industry (Araújo et al. 2012). Usually, a large (preferably very large) initial population is studied under some kind of environment in which potential gains are maximal and stresses reducing those gains are minimal. This is followed by selection cycles with lower strain numbers, but each time more representative of real agricultural usage and ending with field experiments with several cultivars and environmental conditions.

For *Azospirillum*, the first step might be selection under laboratory controlled pure-culture conditions. That *Azospirillum* species fix nitrogen under these conditions is likely why a large part of the older literature attributes to it, at least in part, the field effects of BNF (Dart 1986; Kennedy et al. 1997), although most field studies indicate that its contribution to crop nitrogen status is relatively small (Lana et al. 2012). The next step is generally conducted under some kind of

protected environment such as a greenhouse. There are a large number of papers on this condition, which frequently aim to extend the usage of *Azospirillum* strains to new plant species, to investigate possible mechanisms through which the bacteria affect the plant, or to study a complex mixture of beneficial microorganisms (Viera and Fernandez 2006; Bhattacharjee et al. 2008; Bashan and De-Bashan 2010; Hayat et al. 2010; Jha et al. 2013).

After the protected environment studies, field experiments are most commonly conducted with the major cereals due to their relevance, and their variable results were already mentioned earlier in this section. A large component of this variation may be due to several different mechanisms proposed for *Azospirillum* effects not being adequately adjusted in the experimental design and/or in the results and discussion, leading to an apparent lack of response to the inoculation (Naiman et al. 2009; Pedraza et al. 2009; Hungria et al. 2010; Mostafa and Abo-Baker 2010; Yadegari et al. 2010; Trivedi and Bhatt 2011; Lana et al. 2012; Moghadam et al. 2012; Ferreira et al. 2013; Jha et al. 2013; Perez-Montano et al. 2014). This problem has been mentioned since the 1990s (Okon and Labandera-Gonzalez 1994), and a suggestion given by these authors in their review was a market regulation on inoculant producers.

Under Brazilian law, no commercial inoculant may be used in agricultural fields if not recommended by the Agriculture Ministry (Brasil 2011), which currently recommends *A. brasilense* strains (Table 1) for use in the three major cereal crops of Brazil (wheat, corn and rice). These strains may only be recommended after field trials at several different locations and agricultural years. This legal demand induces a much stronger confidence in the inoculant than may be seen otherwise. One point that should also increase the confidence of the crop grower, with regard to *Azospirillum* inoculant usage, is that most cereal producers also grow soybean and that inoculant usage is widespread and continues to this date (Alves et al. 2003; Phillips 2004).

The very strong adoption of rhizobial inoculants in Brazil for soybean, and to a lesser extent for other legume crops (Zilli et al. 2011), indicates that as long as consistent results can be obtained from *Azospirillum* inoculants under field conditions, we can expect future growth of this biological technique. Another point in which the *Azospirillum* inoculants industry may emulate the rhizobia-based

Table 1 Recommended strains for some cereals designed for use in commercial inoculant production in Brazil (Brasil 2011)

Plant species	Bacterial species	Strain
<i>Triticum</i> spp.	<i>Azospirillum brasilense</i>	Ab-V1
<i>Zea mays</i>	<i>A. brasilense</i>	Ab-V4
<i>Zea mays</i> and <i>Triticum</i> spp.	<i>A. brasilense</i>	Ab-V5
<i>Zea mays</i> and <i>Triticum</i> spp.	<i>A. brasilense</i>	Ab-V6
<i>Zea mays</i>	<i>A. brasilense</i>	Ab-V7
<i>Triticum</i> spp.	<i>A. brasilense</i>	Ab-V8
<i>Oryza sativa</i>	<i>A. brasilense</i>	Ab-V5
<i>O. sativa</i>	<i>A. brasilense</i>	Ab-V6

industry is in the constant pursuit of new technologies for the inoculant, including application forms and liquid formulations, which have caused increased inoculant usage in Brazil in the recent years (Albareda et al. 2008; Vieira Neto et al. 2008; Zilli et al. 2010; França et al. 2013). It must be mentioned that there is already the literature on these aspects including shelf life of the product and the use of commercial products (Sivasakthivelan and Saranraj 2013; Trujillo-Roldán et al. 2013).

An additional problem that must be dealt with is the inoculant's adaptation to field conditions and practices, such as genetically modified organisms (GMO), herbicide usage and seed coating with pesticides, which are major causes of concern for the rhizobial inoculant industry (Austin et al. 2006; Bunemann et al. 2006; Gaiad et al. 2007; Jacques et al. 2010; Zobiolo et al. 2011), but have not been well evaluated up to now.

6 Concluding Remarks

Most studies of the *Azospirillum*-plant association have been conducted on cereals and grasses, while only a few other plant families have been investigated. Recent progress on the understanding of their diversity, colonization ability, action mechanisms, formulation and application of these biological systems should facilitate their development as reliable components in the management of sustainable agricultural. Naturally, the mode of root colonization by *Azospirillum* may vary, depending on the bacterial strain, plant species, environmental conditions and other unidentified factors. Furthermore, the principal mechanism by which *Azospirillum* enhances plant growth is undetermined. However, several possible modes of action have been proposed.

In this regard, efforts have been made by researchers to clearly define and develop commercial inoculants using these organisms with special emphasis on formulations and polymeric carriers. Furthermore, combinations of beneficial bacterial strains that interact synergistically are currently being devised, and numerous recent studies show a promising trend in the field of inoculation technology. The future challenge is to identify management conditions that can contribute to the optimization of several mechanisms of the plant-microorganism interrelationship and that may participate in the association and affect plant growth, including N₂ fixation, hormonal effects, general improvement in root growth and major bio-control activities.

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