



Everything you must know about *Azospirillum* and its impact on agriculture and beyond

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Abstract

Azospirillum is one of the most studied plant growth-promoting bacteria (PGPB); it represents a common model for plant-bacterial interactions. While *Azospirillum brasilense* is the species that is most widely known, at least 22 species, including 17 firmly validated species, have been identified, isolated from agricultural soils as well as habitats as diverse as contaminated soils, fermented products, sulfide springs, and microbial fuel cells. Over the last 40 years, studies on *Azospirillum*-plant interactions have introduced a wide array of mechanisms to demonstrate the beneficial impacts of this bacterium on plant growth. Multiple phytohormones, plant regulators, nitrogen fixation, phosphate solubilization, a variety of small-sized molecules and enzymes, enhanced membrane activity, proliferation of the root system, enhanced water and mineral uptake, mitigation of environmental stressors, and competition against pathogens have been studied, leading to the concept of the Multiple Mechanisms Hypothesis. This hypothesis is based on the assumption that no single mechanism is involved in the promotion of plant growth; it posits that each case of inoculation entails a combination of a few or many mechanisms. Looking specifically at the vast amount of information about the stimulatory effect of phytohormones on root development and biological nitrogen fixation, the Efficient Nutrients Acquisition Hypothesis model is proposed. Due to the existence of extensive agriculture that covers an area of more than 60 million hectares of crops, such as soybeans, corn, and wheat, for which the bacterium has proven to have some agronomic efficiency, the commercial use of *Azospirillum* is widespread in South America, with over 100 products already in the market in Argentina, Brazil, and Uruguay. Studies on *Azospirillum* inoculation in several crops have shown positive and variable results, due in part to crop management practices and environmental conditions. The combined inoculation of legumes with rhizobia and *Azospirillum* (co-inoculation) has become an emerging agriculture practice in the last several years, mainly for soybeans, showing high reproducibility and efficiency under field conditions. This review also addresses the use of *Azospirillum* for purposes other than agriculture, such as the recovery of eroded soils or the bioremediation of contaminated soils. Furthermore, the synthetic mutualistic interaction of *Azospirillum* with green microalgae has been developed as a new and promising biotechnological application, extending its use beyond agriculture.

Keywords *Azospirillum* · Phytohormones · Nitrogen fixation · Plant growth promotion bacteria

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Introduction

Azospirillum is a Gram-negative, microaerophilic, non-fermentative, and nitrogen-fixing bacterial genus. It has been one of the most studied plant growth-promoting bacteria (PGPB) since its discovery by Martinus Beijerinck in the Netherlands in 1925. However, as a result of the research conducted by Johanna Döbereiner in Brazil in the 1970s, two main characteristics are used to define this bacterial genus: its ability to fix atmospheric nitrogen (N) (Day and Döbereiner 1976) and produce several phytohormones, including auxins, cytokinins, and gibberellins (Reynders and Vlassak 1979; Tien et al. 1979). Consequently, in subsequent studies, these two characteristics have been considered the cornerstone of the effect of this genus on plant growth and crops. Because *Azospirillum* is one of the most studied PGPB worldwide, and it has been commercialized in several South American countries, including Argentina, Brazil, Uruguay, and Paraguay (Okon and Labandera-Gonzalez 1994; Cassán and Diaz-Zorita 2016), a significant amount of knowledge has been accumulated, demonstrating different aspects of the plant-bacteria interaction under *in planta* and *in vitro* conditions. It is difficult to identify and quantify the agronomical use of *Azospirillum* in countries other than those in South America. We are aware of products in Mexico, India, China, the United States (US), South Africa, Australia, and France, but no official information is available about the number of hectares (ha) treated, type of crops, type of products, and strains used. Therefore, this review focuses on its use in the South American countries, and several of the available references presenting the data are either in Spanish or Portuguese.

Major changes in the plant root architecture is the main outcome of inoculation with *Azospirillum*. It is generally accepted that these developmental responses are triggered by the production of bacterial phytohormones, and more specifically by the biosynthesis of indole-3-acetic acid (IAA) (Cassán et al. 2014). Despite exhaustive efforts to define a single mode of action to explain the plant growth facilitated by inoculation with *Azospirillum*, the mode is still undefined. However, some hypotheses have been proposed to better understand the benefits of the *Azospirillum*-plant interaction (Bashan et al. 2004; Bashan and de-Bashan 2010). This review aimed to understand the evolution of the research on the agronomical use of *Azospirillum* conducted over the last several decades, and to identify its novel use for environmental purposes and biotechnological applications beyond the agricultural industry. Based on the gathered information and new evidence brought to light in the past several years, a novel hypothesis is proposed to explain the plant growth promotion capability of these bacteria.

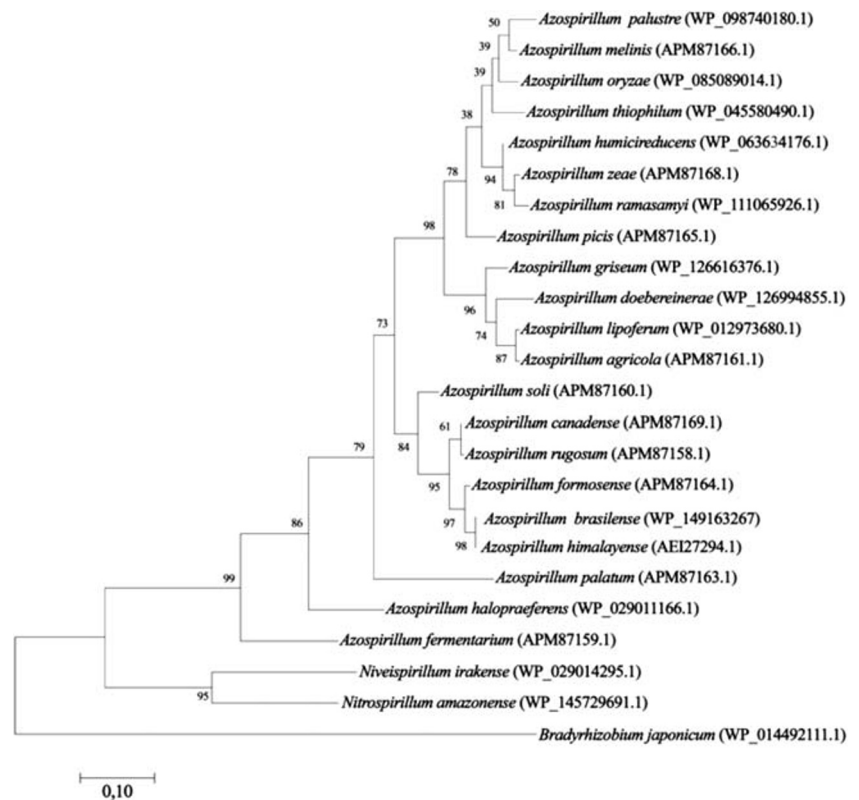
The genus *Azospirillum*

The *Azospirillum* species (*Azospirillum* spp.) are alpha-proteobacteria that are members of the *Rhodospirillaceae*

family (Baldani et al. 2005). While most of the representatives of this family are found in aquatic environments, *Azospirillum* spp. have mainly been isolated from soil. Genomic analysis suggests that, throughout the evolutionary process, this genus transitioned from aquatic to terrestrial environments significantly later than the major Precambrian divergence of hydrobacteria and terrabacteria (nearly 2.5 billion years ago), coinciding with the major radiation of vascular plants on land 400 million years ago (Wisniewski-Dyé et al. 2011). However, for scientists, the history of this genus begins in 1925 when Beijerinck first observed a spirillum-like bacterium isolated from garden soil that was able to increase the N content in nitrogen-deficient malate-based media. Beijerinck initially named the organism *Azotobacter largimobile*; 3 years later, he renamed it *Spirillum lipoferum* (Beijerinck 1925). For 50 years, the importance of this bacterial genus as a research subject decreased until 1974 when its capacity to form a strong association with plant roots was discovered (Von Bülow and Döbereiner 1975). Another fact that awakened interest in these bacteria was their isolation from several types of soil and the roots of grasses and grain crops (Döbereiner et al. 1976). The genus *Azospirillum* was first proposed by Tarrand et al. (1978). Initially, *A. lipoferum* and *A. brasilense* were the only two species described (Tarrand et al. 1978). Since then, as summarized in Fig. 1 and Supplementary Fig. 1, a total of 22 species belonging to this bacterial genus have been identified, including *A. halopraeferens* (Reinhold et al. 1987), *A. largimobile* (Ben Dekhil et al. 1997), *A. doebereineriae* (Eckert et al. 2001), *A. oryzae* (Xie and Yokota 2005), *A. melinis* (Peng et al. 2006), *A. canadense* (Mehnaz et al. 2007a), *A. zea* (Mehnaz et al. 2007b), and *A. rugosum* (Young et al. 2008). Subsequently, new species have been reported: *A. picis* (Lin et al. 2009), *A. palatum* (Zhou et al. 2009), *A. thiophilum* (Lavrinenko et al. 2010), *A. formosense* (Lin et al. 2012), *A. humicireducens* (Zhou et al. 2013), *A. fermentarium* (Lin et al. 2013), *A. himalayense* (Tyagi and Singh 2014), *A. soli* (Lin et al. 2015), and *A. agricola* (Lin et al. 2016). Three new species were identified in 2019: *A. ramasamyi* (Anandham et al. 2019), *A. griseum* (Zhang et al. 2019), and *A. palustre* (Tikhonova et al. 2019). *A. amazonense* (Falk et al. 1985) and *A. irakense* (Khammas et al. 1989) were relocated to separate genera, *Nitrospirillum* and *Niveispirillum*, respectively (Lin et al. 2014).

The genus *Azospirillum* is distributed worldwide and different strains and species have been isolated from several countries, including Argentina, Brasil, China, Taiwan, Korea, Russia, Pakistan, and Irak, among others (Table 1). This genus is considered to be versatile because it has been isolated from different environments (Reis et al. 2015). Although less common, *Azospirillum* spp. have also been found under extreme conditions, such as saline soil, oil-contaminated soil, fermented products, fermentation tanks,

Fig. 1 *Azospirillum* phylogenetic analysis using rpoD sequences obtained from NCBI database. Reference strains of each specie were used. Other members of the *Rhodospirillaceae* family and *B. japonicum* E109 were used as outgroups. Analysis was made by Maximum Likelihood method, Tamura-Nei substitution model, and a Bootstrap testing of 1000 iterations (Jones et al. 1992). Bootstrap values ≥ 50 are shown in the corresponding nodes. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)



sulfide springs, and microbial fuel cells (Reis et al. 2015; Anandham et al. 2019; Tikhonova et al. 2019). Moreover, one member of the *Azospirillum* spp. was isolated from the Himalayan valley and others were found in Baiyang Lake (Reis et al. 2015; Zhang et al. 2019). Nearly 100 years have elapsed since the genus *Azospirillum* was first identified, and the taxonomy information about this type of bacteria continues to grow. Advances in molecular biology allow a better classification of organisms, and the C. C. Young Group from National Chung Hsing University (Taichung, Taiwan) has made the greatest contribution to this research area (Young et al. 2008; Lin et al. 2009, 2012, 2013, 2014, 2015, 2016). Not only have they discovered a significant number of new species and redistributed other species, they have also made significant advances by developing methodologies for the identification of *Azospirillum* strains using polymerase chain reaction (PCR) (Lin et al. 2011).

Functional analysis of plant growth promotion

Azospirillum spp. have been associated to several mechanisms to promote plant growth and a wide range of studies have detailed the beneficial effects of inoculation with these rhizobacteria. The improvement of plant growth by *Azospirillum* spp. has been mostly attributed to their capacity to fix atmospheric N and to produce phytohormones; it is less attributed to the bio-disposition of nutrients, expression of

enzymes, synthesis of compounds related to plant stress mitigation, and competition against phytopathogens, among other mechanisms. However, taken individually, none of these mechanisms has been found to be fully responsible for the changes observed in inoculated plants (Bashan and de-Bashan 2010). *Azospirillum* spp. modes of action were initially explained by the Additive Hypothesis where the effects of small mechanisms operating either at the same time or consecutively create a larger final effect on plants (Bashan and Levanony 1990). In 2010, this hypothesis was replaced by the Multiple Mechanisms Hypothesis, which posits that no single mechanism is involved in the promotion of plant growth; rather, in each case of inoculation a combination of a few or many mechanisms is responsible for the beneficial effect (Bashan and de-Bashan 2010). In the following sections, evidence related to the mechanisms most often studied is summarized to explain the plant growth resulting from inoculation by *Azospirillum* spp.

N fixation

N fixation was the first mechanism to be identified that demonstrated the way in which *Azospirillum* positively affects plant growth (Döbereiner et al. 1976; Okon et al. 1983); therefore, many studies have investigated it and a substantial amount of information about it has been published (Kennedy et al. 2004; Baldani and Baldani 2005; Bashan and de-Bashan

Table 1 Country of origin and source of isolation of *Azospirillum* species

| <i>Azospirillum</i> | Origin ^a | Isolated from ^b | Reference |
|--------------------------|---------------------|---|--------------------------|
| <i>A. lipoferum</i> | Brazil | Wheat roots | Beijerinck (1925) |
| <i>A. brasilense</i> | Brazil | <i>Digitaria decumbens</i> roots | Tarrand et al. (1978) |
| <i>A. halopraeferens</i> | Pakistan | Roots of Kallar grass grown on salt-affected soils | Reinhold et al. (1987) |
| <i>A. largimobile</i> | Australia | Fresh lake water | Ben Dekhil et al. (1997) |
| <i>A. doebereineriae</i> | Germany | <i>Miscanthus sinensis</i> cv. “ <i>giganteus</i> ,” washed roots | Eckert et al. (2001) |
| <i>A. oryzae</i> | Japan | Rhizosphere of <i>Oryza sativa</i> | Xie and Yokota (2005) |
| <i>A. melinis</i> | China | Tropical molasses grass (<i>Melinis minutiflora</i>) | Peng et al. (2006) |
| <i>A. canadense</i> | Canada | Corn rhizosphere | Mehnaz et al. (2007a) |
| <i>A. zeae</i> | Canada | Corn rhizosphere | Mehnaz et al. (2007b) |
| <i>A. rugosum</i> | Taiwan | Oil-contaminated soil near the oil refinery | Young et al. (2008) |
| <i>A. picis</i> | Taiwan | Discarded road tar | Lin et al. (2009) |
| <i>A. palatum</i> | China | Forest soil | Zhou et al. (2009) |
| <i>A. thiophilum</i> | Russia | Bacterial mat of a sulfide mineral spring | Lavrinenko et al. (2010) |
| <i>A. formosense</i> | Taiwan | Paddy soil | Lin et al. (2012) |
| <i>A. humicireducens</i> | China | Microbial fuel cell | Zhou et al. (2013) |
| <i>A. fermentarium</i> | Taiwan | Industrial fermentative tank | Lin et al. (2013) |
| <i>A. himalayense</i> | India | Himalayan Valley soil ^a | Tyagi and Singh (2014) |
| <i>A. soli</i> | Taiwan | Agriculture soil | Lin et al. (2015) |
| <i>A. agricola</i> | Taiwan | Cultivated soil | Lin et al. (2016) |
| <i>A. ramasamyi</i> | Korea | Fermented bovine products ^a | Anandham et al. (2019) |
| <i>A. griseum</i> | China | Water at Baiyang Lake ^a | Zhang et al. (2019) |
| <i>A. palustre</i> | Russia | Sphagnum-dominated raised peatland ^a | Tikhonova et al. (2019) |

^a Information obtained from original report

^b Information obtained from Global Catalogue of Microorganisms [<http://gcm.wfcc.info/>]

2010). The emphasis on this mechanism is due to the significant increase in the total amount of N in shoots and grains observed after *Azospirillum* inoculation in wheat, sorghum, and panicum, among other cereal and grass species (Kapulnik et al. 1981). However, the evidence collected during subsequent decades is controversial. Numerous greenhouse and field experiments demonstrated the contribution of fixed N by bacteria on crops by a reduction in the doses of N fertilizers used under field conditions (for a review, see Bashan and de-Bashan et al. 2010). Incorporation of atmospheric N into the host plant by inoculating with *Azospirillum* was initially evaluated using the acetylene reduction assay (ARA) and later using isotopic ¹⁵N₂ and ¹⁵N-dilution techniques. ARA has contributed to the understanding of *Azospirillum*-gramineae associations, but in its use for definitive quantification of biological nitrogen fixation (BNF), it has many disadvantages, mainly due to the fact that it is a short-term assay of enzyme activity and such activity is drastically reduced when plants are disturbed. While isotope techniques (¹⁵N) have been more popular, they are not easily adaptable under field conditions due to the uniform labeling

of soils and the selection of suitable non-N₂-fixing control plants (Boddey and Knowles 1987). Solid evidence that N fixation contributes to the N balance of plants has been mainly based on the observation of an increase in the nitrogenase activity within inoculated roots with sufficient magnitude to increase the total N yield of the inoculated plants (Bashan and Holguin 1997; Kennedy et al. 1997). However, many studies have shown that the contribution of N fixation by *Azospirillum* to plants is minimal (an increase of 5–18% in the total N of inoculated plants); consequently, plant growth promotion was induced by other mechanisms. These findings almost resulted in the abandonment of the N fixation aspects of *Azospirillum*, except in pure genetic and molecular studies.

In the last years, several studies have focused on N metabolism within bacterial cells, and many details of molecular mechanisms have been studied in *Azospirillum*, which is considered a bacterial model for investigating non-symbiotic N fixation. In this sense, during the genomic era, the Sp245 strain of *A. brasilense* has been used as a model to understand the N metabolism pathways since its genome had been completely sequenced and this strain has been physiologically

characterized. The *nif* gene cluster was identified in two specific positions of the genome; in one case, it was probably codified for an alternative iron or vanadium nitrogenase. The ammonia assimilation in *Azospirillum* occurs via two pathways, one involving glutamate dehydrogenase (*gdhA*) under a high NH_4^+ concentration and the other involving glutamine synthetase (*glnA*) and glutamate synthase (*gltBgltd*) under limiting NH_4^+ . The genes involved in both pathways are present in all the *Azospirillum* species that have been analyzed to date (de Souza and Pedrosa 2015).

Two innovative approaches regarding N fixation research have been developed in the last decades: (a) obtaining the spontaneous ammonium excreting mutants of *A. brasilense* (see Bashan and de-Bashan 2010) and (b) induction of a specialized sites for N fixation on the roots of legume plants known as paranodes. Externally, paranodes resemble a legume nodule and they can be induced in grasses by exogenous application of auxins (Tchan et al. 1991). Under the premise that *Azospirillum* does not secrete significant amounts of ammonium obtained from BNF on plant tissues, *A. brasilense* cells were inoculated into rice and evaluated for their capacity to colonize root paranodes previously induced by treating the roots with auxins. The bacteria colonization of paranodes in the treated plants was correlated with significant increases in plant biomass in comparison to the non-inoculated plants (Christiansen-Weniger and van Veen 1991). Additionally, the nitrogenase activity was significantly higher in the *Azospirillum*-inoculated paranodes of the roots of the rice plants in comparison to the control plants (Christiansen-Weniger 1997). According to Christiansen-Weniger (1997), this was likely because nitrogenase was less sensitive to the oxygen tension in the paranodes than in the rest of the root. Similar increases in nitrogenase activity were reported by Tchan et al. (1991), Zeman et al. (1992), and Yu et al. (1993) in wheat roots containing paranodes colonized by *Azospirillum*. In addition to rice and wheat (Katupitiya et al. 1995), paranodes were also obtained on the roots of maize seedlings (Saikia et al. 2004, 2007).

Machado et al. (1991) characterized a spontaneous mutant, HM053, derived from *A. brasilense* FP2 (Sp7 ATCC 29145, SmR, NaIR), which was resistant to ethylenediamine (EDAR). This mutant was able to excrete ammonium and fix N in the presence of high concentrations of NH_4^+ ; hence, it is an interesting candidate for use as a biofertilizer to supply N to gramineaceous plants. Machado et al. (1991) suggested that the mutant HM053's ability to excrete ammonium is related to low glutamine synthetase activity, resulting in a deficiency of NH_4^+ assimilation; this explains the excretion of excess ammonium produced during N fixation. Pankievicz et al. (2015) showed that *Setaria viridis* inoculated with the HM053 strain incorporates a significant N level via BNF, and this level may be enough to provide the plant's daily N demand. Moreover, HM053 was able to promote wheat and

barley growth (Santos et al. 2017) and *nif* expression in *planta* during wheat root colonization, which was shown to be about 300-fold higher growth than with the wild type strain. The same strain outperformed the parental strain in field experiments, leading to a maize yield increase of up to 28% (Pedrosa et al. 2019). Similar ammonium excreting mutants of *A. brasilense* have been reported to enhance plant growth (Van Dommelen et al. 2009). Moreover, some of the mutants have been evaluated using the paranodes colonization system (Christiansen-Weniger and Van Veen 1991).

Phytohormone production

Due to evidence reported in studies published over the past 90 years, it is known that the *Azospirillum* genus is associated with the production of several phytohormones. Simultaneously, Reynders and Vlassak (1979) and Tien et al. (1979) reported the capacity of *Azospirillum* to produce indole-3-acetic acid (IAA) under in vitro and in vivo conditions, respectively. Additional investigations revealed the capacity to produce cytokinins (Tien et al. 1979), gibberellins (Bottini et al. 1989), ethylene (Strzelczyk et al. 1994), and other plant growth regulators, such as abscisic acid (ABA) (Kolb and Martin 1985), nitric oxide (Creus et al. 2005), and polyamines, such as spermidine, spermine, and the diamine cadaverine (Thuler et al. 2003; Cassán et al. 2009). The plant growth regulators and phytohormones produced by *Azospirillum* have been summarized and ranked according to their effects on plants in previous reports (see Table 1, Cassán and Diaz-Zorita 2016). In a culture medium, the concentrations of the most important groups of plant hormones produced by this bacterium, such as auxins, cytokinins, and gibberellins, increase with bacterial growth because these compounds are continuously accumulated in the medium according to a batch fermentation model (Ona et al. 2003; Cassán et al. 2009; Molina et al. 2018). Based on the active principles of inoculants, both the bacteria (cell number) and the metabolites (mainly phytohormones) are biosynthesized, released, and accumulated in the culture medium. Then, inoculants with a different metabolite profile should have a different capacity to promote the growth of inoculated plants, even if the number of cells is equal. In the case of seed inoculation, the use of inoculants containing *Azospirillum* and phytohormones in the culture medium will produce a “seed priming” effect. In this sense, Okon (1982) reported that, after seed inoculation, the number of viable *Azospirillum* cells decreases very rapidly. Then, the short-term benefits of seeds inoculation should not be strictly related to the presence of the bacterial cells in the inoculant; instead, they are, at least partly, related to the presence and concentration of several phytohormones and plant growth regulators. This has been defined as the hormonal effect of inoculation (Cassán et al. 2014).

Auxin metabolism

Auxins are a group of plant growth regulators that are involved in numerous aspects of plant growth and development (Teale et al. 2006). IAA is the predominant plant growth regulator found in plants. It is acknowledged that 80% of rhizobacteria, including *Azospirillum*, are able to produce IAA and the synthesis pathways are similar to those found in plants (Spaepen et al. 2007). At present, members of the genus *Azospirillum* have provided an excellent experimental model for investigating the physiological role of auxins in PGPB-plant interactions, and several naturally occurring auxin-like molecules have been described as products of bacterial metabolism. The genome sequence of *A. brasilense* Az39 revealed the existence of all the genes involved in the indole-3 pyruvate (IPyA) pathway: *hisC1* coding for an aromatic amino transferase, *ipdC* coding for an indole-3-pyruvate decarboxylase, which is considered to be the key enzyme of this pathway (Broek et al. 1999), and an aldehyde dehydrogenase gene (see Table 1, Cassán et al. 2014). For the Sp245 and CBG497 strains, only the *hisC1* and *ipdC* genes were identified; no evidence of aldehyde dehydrogenase was observed in these genomes. Considering that the genome sequences of *A. brasilense* Sp245 and Az39 are very similar, it is not surprising that all the genes encoding for the IPyA pathway are very similar in both strains. No evidence has been found for the existence of *ipdC* or aldehyde dehydrogenase in the genome sequence of *A. lipoferum* 4B. Only a putative aromatic amino transferase sequence with homology to AAT1 from *A. brasilense* Sp7 has been identified (Wisniewski-Dyé et al. 2011). *Azospirillum* sp. B510 genome sequence analysis revealed a putative aromatic amino transferase with homology to AAT1 from *A. brasilense* Sp7 (Wisniewski-Dyé et al. 2011). Kaneko et al. (2010) proposed that two candidate genes are involved in the indole acetamide (IAM) pathway, but we question their role in IAA biosynthesis due to the low similarity (especially for the putative *iaaM* gene) between them and the known *iaaM* and *iaaH* genes. Finally, gene encoding nitrilases have also been identified in the *Azospirillum* sp. B510 genome (Wisniewski-Dyé et al. 2012).

In addition to IAA, other molecules, such as indole-butyric acid (IBA) (Martínez-Morales et al. 2003), phenyl acetic acid (PAA) (Somers et al. 2005), indole-3-lactic acid (ILA), indole-3-ethanol and indole-3-methanol (Crozier et al. 1988), indole-3-acetamide (IAM) (Hartmann et al. 1983), indole-3-acetaldehyde (Costacurta et al. 1994), tryptamine (TAM), and anthranilate (Hartmann et al. 1983), have been identified in an *Azospirillum* spp. culture medium. At least four different IAA biosynthesis pathways have been proposed in *Azospirillum* spp.: the tryptophan-dependent pathways IPyA, IAM, and TAM, and a putative tryptophan-independent pathway (Prinsen et al. 1993). Despite this diversity, IPyA is considered to be the most important pathway for IAA

biosynthesis in this genus. The question about why some bacteria are able to produce phytohormones remains unanswered; however, in the case of auxins, a co-evolutionary mechanism could be hypothesized. Plants release several compounds, such as amino acids and organic acids, into the rhizosphere through root exudates. In the case of amino acids, and particularly for L-trp, this precursor could be used by auxin-producing bacteria to biosynthesize IAA. This molecule increases the amount of this hormone in the rhizosphere, which induces changes in the plant, increasing its root morphology and growth. Thus, a higher amount of root exudate in the rhizosphere will increase the availability of nutrients for the bacteria living in the rhizosphere, enhancing their population. Higher levels of IAA in the rhizosphere will induce a higher *ipdC* gene expression by *Azospirillum*, thereby enhancing the IAA concentration in the rhizosphere and stimulating root growth. In other words, some bacteria are able to increase their own population within the rhizosphere by producing IAA using the L-trp produced by plants as a co-evolutionary mechanism. How do plants regulate the IAA levels in the rhizosphere? This should be the most important question for this model; the answer is related to the ability of the plant to regulate the release of L-trp and other amino acids in the rhizosphere by the exudates. In this sense, the full IAA metabolism of *A. brasilense* has been recently revealed (Rivera et al. 2018). Rivera et al. (2018) found that some amino acids, such as L-met, L-val, L-cys, and L-ser, inhibit bacterial growth and reduce IAA biosynthesis, while the expression of *ipdC* and IAA biosynthesis, but not bacterial growth, are affected by L-leu, L-phe, L-ala, L-ile, and L-pro. Furthermore, L-arg, L-glu, L-his, L-lys, L-asp, and L-thr do not affect bacterial growth, IAA biosynthesis, or *ipdC* gene expression; this fact should have some impact on the rhizosphere during plant-microbe interactions (see Fig. 2, Rivera et al. 2018). It was also confirmed that the *A. brasilense* strains Sp245, Az39, and Cd can only produce IAA in the presence of L-trp (biosynthesis); these strains are unable to degrade auxins (catabolism), conjugate IAA with sugars and/or L-amino acids (conjugation), or hydrolyze conjugates to release free IAA (hydrolysis). IAA biosynthesis was also evaluated under abiotic and biotic stress conditions; it was found to increase with daylight or in the presence of PEG₆₀₀₀, ABA, salicylic acid (SA), chitosan, and a filtered supernatant of *Fusarium oxysporum*. In contrast, exposure to 45 °C or treatment with H₂O₂, NaCl, Na₂SO₄, 1-aminocyclopropane 1-carboxylic acid, methyl jasmonate, and a filtered supernatant of *Pseudomonas savastanoi* decreases IAA biosynthesis (Molina et al. 2018).

Root growth phytostimulation

Roots are the plant organs that are preferentially modified by *Azospirillum* (see Bashan and de-Bashan 2010). In the 1990s, enhanced water and mineral uptake by roots was frequently

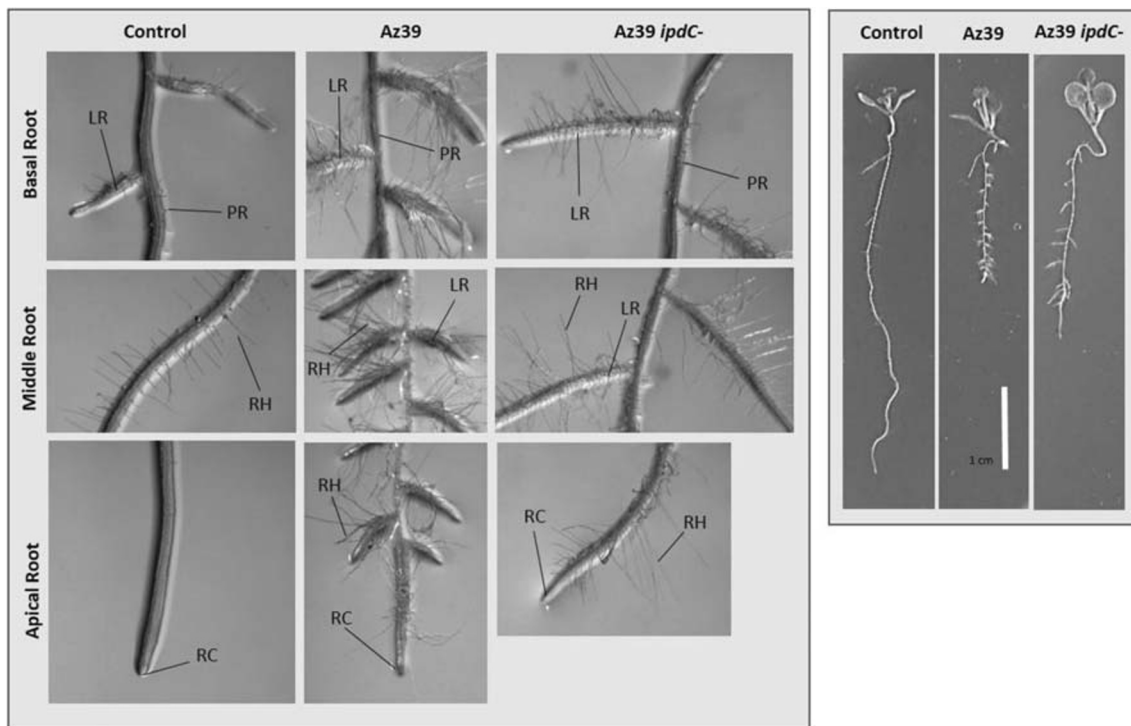


Fig. 2 Changes in the architecture of the 12-day-old seedling root system of *Arabidopsis*. Seedlings were grown for 7 days on MS medium, then were inoculated for 5 days with *A. brasilense* Az39 or Az39 *ipdC*-mutant

deficient in IAA production. Images show different zones of the root: basal, middle, and apical root. Primary root (PR), lateral roots (LR), hair root (HR), root cap (RC). Photography credits: Mora V

used to explain the beneficial effects of *Azospirillum* inoculation (see Bashan and Levanony 1990; Bashan and Holguin 1997). Increased mineral uptake and water absorption have been related to changes in root growth, architecture, and volume instead of any specific metabolic enhancement process (Murty and Ladha 1988). This fact has been strictly related to the bacterial capacity to produce phytohormones. However, the descriptive data presented thus far have not shown whether these improvements are the cause or the result of other mechanisms (Bashan and de-Bashan 2010). The first evidence of phytostimulation by *A. brasilense* was observed in pearl millet and sorghum seedlings, and it was similar to that observed by exogenous application of IAA (Tien et al. 1979). Later, it was shown that inoculation of *Beta vulgaris* increased the number of lateral roots in the inoculated plants in comparison to the uninoculated plants. This effect was correlated with the high levels of IAA produced by bacteria in a pure liquid culture medium and it was mimicked by the exogenous addition of similar concentrations of the phytohormone (Kolb and Martin 1985). The current model of root growth phytostimulation by *Azospirillum* includes a number of morphological changes that can be summarized as follows: (1) decrease in the elongation of the main root (Dobbelaere et al. 1999; Spaepen et al. 2007); (2) increase in the lateral and adventitious roots (Fallik et al. 1994; Molina-Favero et al. 2008); (3) increase in the number of root hairs (Okon and Kapulnik 1986; Hadas and Okon 1987); (4) branching of the root hairs (Jain and Patriquin

1985); and (5) significant increase in the root surface and volume, probably related to the improvement in water and nutrient acquisition (Spaepen et al. 2014). Modifications in the root architecture mediated by *Azospirillum* have shown that there is an IAA-dependent response to inoculation. However, recent evidence suggests that other molecules or cell components would be able to induce an IAA-like response to inoculation (IAA independent response). In this sense, and as shown in Fig. 2, *A. brasilense* Az39 is able to induce the typical root phytostimulation effect in *Arabidopsis thaliana* under in vitro conditions due to IAA production. However, inoculation with *A. brasilense* Az39 *ipdC*- (a non-IAA producer mutant) still induced a stimulatory effect similar to the one induced by IAA on *Arabidopsis* roots (V. Mora, personal communication). This result increases the complexity of the current model and forces us to work with alternative hypotheses to establish the definitive model, which, in spite of many published papers and a significant amount of effort, has not yet been finalized.

The stimulation of plant root growth by *Azospirillum* induces an increase in the water absorption and nutrient acquisition rates (including N), which clearly improves the assimilation of N in the biomass and, more generally, plant growth. This capacity would be mediated by the bacterial colonization of the roots and/or their ability to produce different phytohormones, mostly during early stages of plant development. Consequently, the increase in the root biomass would

increase the supply of root exudates into the rhizosphere, which would increase the bacterial population associated with the roots and improve their ability to colonize this organ and the rest of the plant. Once the plant is colonized with a high number of bacteria, e.g., $> 10^5$ cfu g⁻¹ according to Okon (1982), these cells would be able to provide the plant with significant amounts of NH₄⁺ via BNF. During the advanced stages of plant development, this would have a greater impact on the N economy for the plant. In summary, the Efficient Nutrients Acquisition Hypothesis by inoculated plants would depend on both biological N fixation and phytohormone biosynthesis by the effectively colonized bacteria.

The impact of *Azospirillum* inoculation on agriculture

Worldwide, the market of inoculants containing *Azospirillum* spp. is flourishing in South America. Here, the inoculation was initially focused on cereal production, but nowadays, and mostly in Brazil, inoculation is additionally focused on legumes, such as soybeans, combining it with rhizobia inoculants (co-inoculation). The changes in plant growth observed by *Azospirillum* inoculation and the bacterial capacity to improve the negative effects of abiotic stress on crops has attracted the attention of researchers interested in developing field applied studies (Okon and Labandera-Gonzalez 1994). Okon et al. (2015) suggested that because the diverse modes of action of *Azospirillum* mostly stimulate plant root growth, inoculation with this microbe could contribute to the increase and stabilization of crop production. However, evaluations of the efficacy of *Azospirillum* under current crop management practices and at regular environmental conditions are scarce and have been conducted on different crops and in different regions.

Based on 347 trials obtained from 12 countries, including Brazil, Argentina, and several countries in Southeast Asia, and 47 published articles, mainly focusing on maize and other cereals, the impact of *Azospirillum* inoculation has been analyzed (Díaz-Zorita et al. 2015). From this analysis, the greatest contribution of *Azospirillum* inoculation to grain yield was observed in winter cereals followed by summer cereals and other crops (Fig. 3). The reviewed studies on inoculation with *Azospirillum* showed variable results and a multiplicity of interactions related not only to crop management practices but also to environmental conditions. Most field assays have been performed in single geographical locations during one or two consecutive seasons. Thus, of the ability to analyze the performance of bacterial inoculation under random temporal and spatial conditions is limited.

Based on a total of 316 field experiments performed in the pampas region (Argentina), the relative yield increase in maize due to inoculation with *A. brasilense* showed positive results, ranging between 66 and 80% of positive responses in comparison with untreated control (Díaz-Zorita et al. 2015).

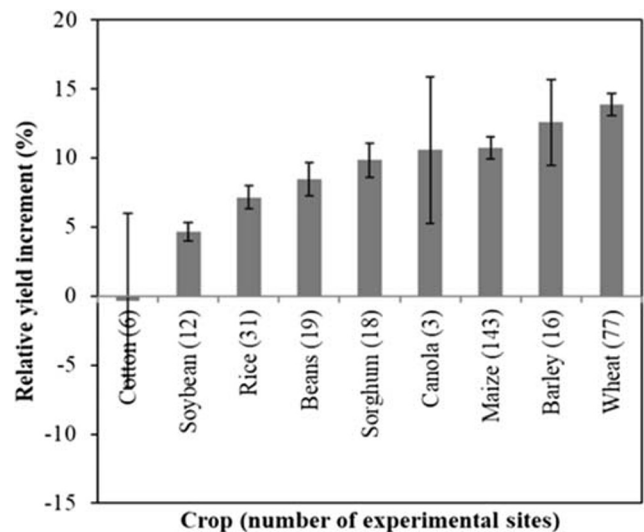


Fig. 3 Mean contribution of the inoculation with *Azospirillum* sp. on crop grain production reviewed from 47 worldwide published field trials under regular production practices. (Adapted from Díaz-Zorita et al. 2015)

Among the growing seasons, the relative contribution of *Azospirillum* to maize yield increases under conditions with less rainfall during the early growth stages (Supplementary Fig. 2). In wheat, the early season effects of inoculation with *Azospirillum* decrease if favorable growing conditions occur during the seed filling stage (Kazi et al. 2016). Okon and Labandera-Gonzalez (1994) and Díaz-Zorita and Fernández-Canigia (2009) found that the grain production responses to inoculation with *Azospirillum* spp. in wheat and other crops were successful in 70–80% of the cases, regardless of the production conditions. In part, this behavior is caused by the complexity of the impact of *Azospirillum* on plants interacting with the impact of several abiotic stressful conditions. Kazi et al. (2016) reported that azospirilla inoculation increased the bacteria population in the rhizosphere during the early stages of growth. Most of the benefits have been observed during the early growth stages of plants with greater and more consistent responses seen in the root and shoot dry matter production and a minimal contribution to the grain yield components during the seed filling period (Díaz-Zorita and Fernández-Canigia 2009; Veresoglou and Menexes 2010). Based on the analysis of 480 greenhouse and field experiments, Veresoglou and Menexes (2010) validated the benefits of wheat inoculation with *Azospirillum*, but they considered variable responses based on differences in the management practices, such as N fertilization, wheat genotype, or *Azospirillum* strain, that were used to inoculate the crops. Although N fertilization benefits wheat production (Saubidet et al. 2002), the relative contribution of *Azospirillum* decreased as the dose of the N fertilizer increased. Under high N availability, the bacterial response was not observed (Ozturk et al. 2003).

The combined inoculation of legumes with rhizobia and azospirilla, defined as co-inoculation, could improve plant

performance due to the complementary nature of the mechanisms of both bacteria. In soybean crops, co-inoculation resulted in both early initiation of nodule ontogenesis and an increase in the number of nodules, thereby increasing the concentration of N in the shoots and improving the plant growth, particularly under drought conditions (Chibeba et al. 2015; Cerezini et al. 2016). Although the contribution of co-inoculation to the productivity of diverse legume crops is promising, the available information about its use under large production conditions is limited. The results from 21 field trials with alfalfa performed in the pampas region (Argentina) showed that the seed treatment combining *Ensifer meliloti* and *A. brasilense* resulted in a response that was nearly two times better than the response obtained from a single inoculation with rhizobia (Díaz-Zorita 2012). Hungria et al. (2013) also reported an increase in grain yields in soybeans and common beans (*Phaseolus vulgaris*) when combining rhizobia seed inoculation with in-furrow application of *A. brasilense* at four sites in Brazil. The single inoculation of *Bradyrhizobium* in soybeans resulted in mean grain yield increases of 8.4% in comparison to the uninoculated control, whereas co-inoculation with *Bradyrhizobium* and *A. brasilense* resulted in an increase of 16.1%. For common beans, the single inoculation with *R. tropici* increased the yield by 8.3%, and co-inoculation of *R. tropici* and *A. brasilense* improved the yield by 19.6% (Hungria et al. 2013). The mean soybean yields from 37 field trials under regular management when *Bradyrhizobium* was co-inoculated with *A. brasilense* were 227 kg per ha greater than when the soybeans were inoculated with *Bradyrhizobium* alone and 335 kg per ha greater than the uninoculated control (Nogueira et al. 2018). The mean effects of co-inoculation on soybean nodulation were evaluated under 22 regular crop production conditions; the results showed differences in the effects between tropical-subtropical and temperate environments. On average, the percentage of soybean nodulation increased by around 5% at the Brazilian sites (Hungria et al. 2015; Fipke et al. 2016; Galindo et al. 2018) and around 12% at the Argentinian sites (Benintende et al. 2010; Ferraris and Couretot 2011, 2013; Morla et al. 2019). However, opposite results were found for grain yields. This limited dataset was insufficient to show a consistent and direct relationship between the use of co-inoculation and changes in nodulation and grain yield. Currently, the use of azospirilla inoculants in Brazil is increasing due to co-inoculation. In the state of Parana (Brazil), the use of co-inoculation between 2016 and 2018 increased by almost 30% (Prando et al. 2016, 2018).

Alternative methods of inoculation that are as effective as the standard seed inoculation technique may represent an important strategy to avoid the incompatibility that can occur between the inoculants and pesticides used during seed treatment. However, these technologies need to be thoroughly evaluated before promoting their extensive use.

Fukami et al. (2016) described the beneficial effects of spraying leaves with *Azospirillum* at the beginning of the vegetative phase. Morais et al. (2016) observed that seed furrow inoculation also increased the maize grain yield under current Brazilian production practices. The benefits of foliar inoculation with *A. brasilense* were evaluated and explained using an auxin signaling model (Puente et al. 2017). The results confirmed soybean growth promotion after seed treatment with *B. japonicum* and foliar co-inoculation with the IAA producer *A. brasilense* Az39. Both auxin production and *A. brasilense* colonization were responsible, via plant signaling, for the positive effects on plant growth and the symbiosis establishment (see Fig. 5 in Puente et al. 2017). An improvement in the nutritional quality of soybean grain due to foliar inoculation with *A. brasilense* Az39 under greenhouse and field conditions was reported 1 year later (Puente et al. 2018). These findings provide new insights into soybean agricultural technology.

Inoculants formulated with *Azospirillum* in South America

Currently, the use of azospirilla inoculants for crop production is a consolidated practice in South America (i.e., Brazil, Argentina, Uruguay, and Paraguay), where the extensive agriculture is frequent (Cassán and Díaz-Zorita 2016). In Argentina, Uruguay, and Brazil, there many biological products contain *Azospirillum* as an active principle. However, the first inoculant in the region was registered 23 years ago (1996) in Argentina with the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) using the name of Nodumax-L by Laboratorios Lopez SRL (Jesús María, Córdoba). It was formulated with *A. brasilense* Az39, one decade after the isolation and selection of this strain by Enrique Rodríguez Cáceres from the Instituto Nacional de Tecnología Agropecuaria (INTA). The inoculant was initially recommended for the treatment of wheat and maize seeds, but it is now recommended for several crops. In Brazil, paradoxically, the first inoculant was registered by Stoller do Brasil SA (Campinas, São Paulo), 14 years after the first one was registered in Argentina. It was named Masterfix L gramineas, and it was formulated with a combination of the *A. brasilense* Abv5 and Abv6 strains. This product was initially recommended for the treatment of maize and rice seeds, but in the last several years, it has also been recommended in combination with *B. japonicum* for soybean co-inoculation. Finally, in Uruguay, the first inoculant product was registered in 2015 by Lage y CIA SA (Montevideo, Montevideo) under the name Graminosoil. It contains a combination of *A. brasilense* Az39 and CFN535. The product was initially recommended for the treatment of maize and sorghum. Currently in South America, there are 106 products (inoculants) produced by 74 companies representing 79 commercial brands. Most of them are

produced in Argentina (90 products); 14 products are produced in Brazil and two products are produced in Uruguay. All the available products for commercialization in the Argentinian market are produced in Argentina, but in Brazil and Uruguay, the inoculants are either locally produced or imported from Argentina. All of the products (100%) are formulated with *A. brasilense*, and the Az39 strain is the active principle in 75% of these inoculants (79 products). In 13 products, Az39 is combined with other *A. brasilense* strains (one product containing CFN535), *Pseudomonas fluorescens* (one product), or *B. japonicum* (11 products). In the last case, this is due to the increase in the number of products registered as a premium technology (co-inoculation) for soybeans. The combination of the *A. brasilense* Abv5 and Abv6 strains is used to formulate 18 products and the combination of the *A. brasilense* Az78 and Az70 strains is used to formulate three products. The rest of the azospirilla inoculants are formulated with single strains (Abv5, AzM3, AzT5, 1003, Tuc 27/85, Tuc 10/1, and 11005). Liquid carriers are most often used to formulate these biological products (94%); 6% of the products are formulated on solid carriers, such as peat or bentonite. In 2014, 82% of the formulations in the market were liquid carriers and 18% were solid carriers. This clearly shows the formulation preferences of the companies that are manufacturing these products. The most frequent shelf life of the registered products is approximately 6 months from production with a minimal concentration of 1×10^7 cfu ml⁻¹ in Argentina or 1×10^8 cfu ml⁻¹ in Brazil and Uruguay. Although the use of these biological products has been recommended for 16 types of crops, the registration is mainly for wheat (67), maize (65), sunflowers (16), and soybeans (12). The other plant species recommend for the treatment with *A. brasilense* are sorghum (*Sorghum bicolor*) (9), grasses, and winter cereals for grazing (4), rice (5), barley (3), cotton (*Gossypium hirsutum*) (3), oats (*Avena sativa*) (2), sugar cane (*Saccharum officinarum*) (1), tobacco (*Nicotiana tabacum*) (1), and lettuce (*Lactuca sativa*) (1). In Brazil, most of the commercialized products are allocated in the maize and soybean grain production market. Based on 2018 data, approximately 7.0 million doses of azospirilla inoculants were commercialized, covering almost 5.0 million ha in South America. In 2014, 3 million ha of plants were inoculated with *A. brasilense* corresponding to 3.5 million doses of these products. This shows a clear trend in the region of increased use of products formulated with these bacteria.

Extending the use of *Azospirillum* beyond the agricultural industry

In addition to its proven usefulness in agriculture, *Azospirillum* possesses the potential to solve environmental problems, such as preventing soil erosion by improving the growth of plants on barren and degraded lands that have lost their capacity to

support regeneration, and participating in phytoremediation strategies to decontaminate soils, all leading to healthier environments (de-Bashan et al. 2012). Although these uses are not yet widespread, some examples are presented in this section.

Puente and Bashan (1993) demonstrated that *A. brasilense* inoculated on the cardon cactus, *Pachycereus pringlei*, the world's largest cactus that stabilizes topsoil in its usual scrub habitat in the Sonoran Desert (Mexico), improves the growth characteristics of the plant. In a field trial, three species of cacti inoculated with *A. brasilense* had a significantly higher survival rate in comparison to the non-inoculated controls. The most important outcome from this trial was the significant reduction in soil erosion and the reclamation of topsoil (Bashan et al. 1999). Growth chamber experiments have demonstrated that *A. brasilense* enhances enzymes in the phosphogluconate pathway and facilitates the growth of mesquite seedlings (*Prosopis articulata*) that are cultivated in poor soils (Leyva and Bashan 2008). In a greenhouse environment, the effect of *A. brasilense* combined with *Bacillus pumilus*, unidentified arbuscular mycorrhizal (AM) fungi (mainly *Glomus* spp.) and compost, were measured on the growth of leguminous trees, such as mesquite, yellow palo verde (*Parkinsonia microphylla*), and blue palo verde (*Parkinsonia florida*), used in desert reforestation and urban gardening in arid northwestern Mexico and the southwestern region of the US (de-Bashan et al. 2012). The mesquite and yellow palo verde had different, positive responses to several parameters, while blue palo verde did not respond (de-Bashan et al. 2012). Later, seven field trials were undertaken with cardon cacti and the same species of leguminous trees (Bashan et al. 2012). The trial showed that, a decade later, a combination of a legume tree with a cardon cactus, while detrimental to the legume, significantly increased the chances of the cactus surviving and growing in degraded soil. (Moreno et al. 2017). Recently, inoculation of *Brachiaria* spp. with *A. brasilense* demonstrated the potential for successful reclamation of degraded pastures in Brazil (Hungria et al. 2016).

In terms of phytoremediation, *A. brasilense* improved the growth of the shrub quailbush, *Atriplex lentiformis*, and affected the rhizosphere microbial community in acidic, metalliferous tailings in Arizona (de-Bashan et al. 2010). Tugarova et al. (2013) proved the capacity of *A. brasilense* strains to reduce selenium (IV) to selenium (0), indicating the possibility of applying *Azospirillum* as a microsymbiont for the phytoremediation of selenium-contaminated soils; moreover, the bioremediation potential of *Panicum virgatum* (switchgrass), along with AM fungi and *Azospirillum*, was tested against lead and cadmium in pot trials (Arora et al. 2016).

In 2000, the Yoav Bashan research group began an interesting study to investigate extending the use of *Azospirillum* from agricultural plants to aquatic green microalgae (Gonzalez and Bashan 2000). Specifically, they created a synthetic mutualism between the microalga *Chlorella* spp. and

A. brasilense, and they proposed it as a simple, quantitative experimental model to study the beneficial interactions between the plant and the bacteria (Fig. 4). To facilitate the interaction and maintain the mutualistic associations, the two microorganisms were initially immobilized in small alginate beads (de-Bashan and Bashan 2008). The hypothesis behind proposing such an interaction was that, as an unspecified PGPB, *A. brasilense* would affect green microalgae in ways that were similar to how it impacted higher plants. They found that the effects occurred at all levels, presenting a new avenue for the application of *A. brasilense*.

Thus far, physiological studies have shown the effects of *A. brasilense* on microalgae pigments (de-Bashan et al. 2002), carbohydrates (Choix et al. 2012a, b, 2018), total lipids (Leyva et al. 2015), and vitamins (Palacios et al. 2016). Similar to higher plants, the production of IAA is a key mechanism affecting microalgae (de-Bashan et al. 2008a). *Azospirillum* enhances the growth of *Chlorella* spp., *Scenedesmus obliquus*, and *Chlamydomonas reinhardtii* (de-Bashan and Bashan 2008; Choix et al. 2018), but it also affects the activities of enzymes, including glutamine synthetase and glutamate dehydrogenase in *C. vulgaris*. A higher uptake of N from the culture medium and a higher accumulation of intracellular N were observed in the plants inoculated with *Azospirillum* than those that were not inoculated (de-Bashan et al. 2008b; Meza et al. 2015). Similarly, it was found that *Azospirillum* had an effect on ADP-glucose pyrophosphorylase, leading to increased accumulation of starch (Choix et al. 2014) and on acetyl-CoA carboxylase, resulting in higher synthesis of fatty acids (Leyva et al. 2014) in microalgae. A direct exchange of N and C between *A. brasilense* and *C. sorokiniana* was demonstrated by nanoSIMS (de-Bashan et al. 2016), and the positive effect of the volatile compounds produced by *A. brasilense* in

C. sorokiniana was also reported (Amavizca et al. 2017). Lopez et al. (2019) showed that riboflavin and lumichrome produced by *A. brasilense* had a significant effect on photosynthetic and auxiliary pigments in *C. sorokiniana*. The combination has been successfully used for wastewater treatment (de-Bashan et al. 2002; Bashan et al. 2004; Perez-Garcia et al. 2010) and recovery of desert degraded soil after amendment of wastewater debris (Trejo et al. 2012; Lopez et al. 2013).

Overall, these results have extended the use of *Azospirillum* beyond agriculture to tackling environmental issues, such as revegetation, reforestation, phytoremediation, and wastewater treatment programs.

An overview of the research timeline

Over the last 90 years, studies on *Azospirillum*-plant interaction have suggested a wide range of mechanisms through which the bacterium enhances plant growth, as summarized in Supplementary Fig. 1.

Despite this body of evidence, two main mechanisms have defined this genus as a model of PGPB: BNF and phytohormone production. The history of the effects of *Azospirillum* as a bacterium capable of fixing atmospheric N dates to 1976 in Brazil. It was revealed for the first time that *A. lipoferum* was able to efficiently fix N in the roots of *Digitaria decumbens* (Day and Döbereiner 1976). This mechanism lost its research importance because the results obtained in greenhouse and field experiments were controversial; however, new mechanisms were proposed to explain the positive effects of inoculation. That was how, at the Katholieke Universiteit Leuven (Belgium), it was demonstrated for the first time that tryptophan was involved in IAA production since *A. brasilense* was able to convert tryptophan into IAA (Reynders and Vlassak 1979). Meanwhile, a study conducted in the US reported that

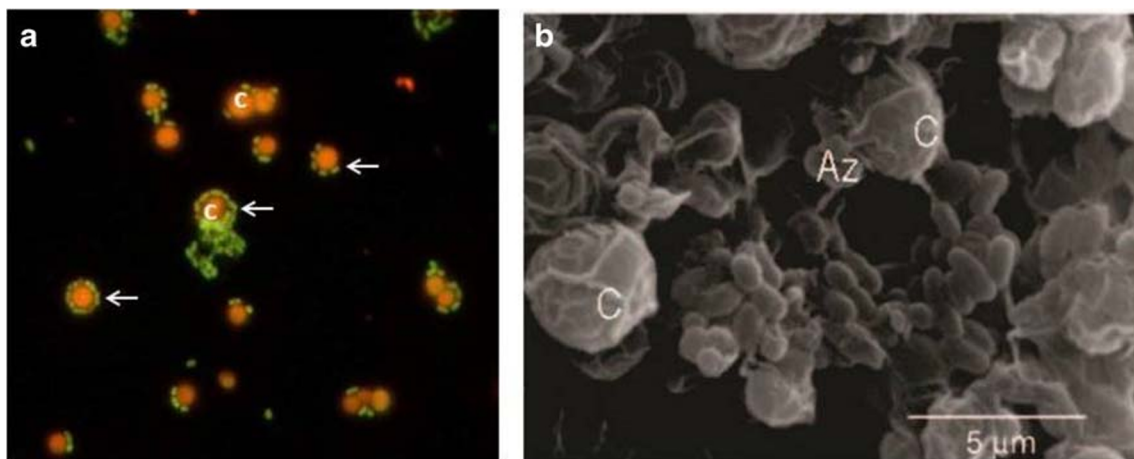


Fig. 4 *Chlorella sorokiniana* and *Azospirillum brasilense* in co-culture. **a** Auto-fluorescence of microalgae appears in orange while bacteria appears in green, as result of fluorescent in situ hybridization (FISH) using three specific probes targeting Eubacteria (FAM dye) and one specific probe for

A. brasilense (CY3 dye). **b** Scanning electron microscopy (SEM). **c** *C. sorokiniana*. **Az**: *A. brasilense*. Arrows show cells of *A. brasilense* attached to the microalgae

A. brasilense was able to produce plant growth substances, such as auxins, cytokinins, and gibberellins (Tien et al. 1979). These two reports were the first to show that *Azospirillum* had the ability to improve plant growth due to the production of phytohormones. Two years later, Oliveira and Drozdowicz (1981) demonstrated for the first time the ability of the genus *Azospirillum* to produce bacteriocins (molecules able to inhibit bacterial growth) in a pure culture medium. One year later, Reynders and Vlassak (1982) investigated the use of *A. brasilense* as a biofertilizer in intensive wheat cropping. Simultaneously, a selective culture medium, Congo Red medium (CR), was developed to isolate *Azospirillum* spp. from soil or seeds (Rodríguez Cáceres 1982).

As the interest in the phytohormonal effects of *Azospirillum* in plants intensified, numerous field studies were conducted in the 1980s to analyze the growth and yield of inoculated crops. Thus, *Azospirillum* began to emerge as a powerful crop inoculant in co-inoculation systems. The first co-inoculation studies were conducted using *A. brasilense* and the mycorrhizal fungus, *Glomus mosseae*, to study their effects on the growth and nutritional quality of maize and ryegrass (Barea et al. 1983). Furthermore, co-inoculation of *Azospirillum* and *Rhizobium* was found to have a positive effect on winged beans and soybeans (Iruthayathas et al. 1983). Sarig et al. (1984) reported that the best effects on plants inoculated with *Azospirillum* were obtained when the culture conditions were sub-optimal. It was first found that the grain yield of non-irrigated *Sorghum bicolor* increased under abiotic stress when inoculated with *Azospirillum* (Sarig et al. 1984). As interest in the phytohormonal effects of these bacteria continued, it was observed that *A. brasilense* was able to produce ABA in a chemically defined culture medium (Kolb and Martin 1985). The production of plant growth substances (phytohormones), classified as cytokinins, by *Azospirillum* and other related bacteria continued to be analyzed (Horemans et al. 1986). Four years later, gibberellins A₁, A₃, and iso-A₃ were identified in cultures of *A. lipoferum* (Bottini et al. 1989). Similar results were obtained using *A. brasilense* (Janzen et al. 1992). Later, ethylene production by *Azospirillum* was evaluated in chemically defined media modified with the amino acid L-methionine (Strzelczyk et al. 1994).

The arrival of the molecular biology and genomics era shifted the focus to investigating the functional effects of *Azospirillum* on plants at the molecular level. The first studies to emerge focused on *Arabidopsis* plants as a model to investigate the *A. brasilense*-*Arabidopsis* root interaction system; they demonstrated that this bacterium more than doubled the root hair growth in comparison to the non-inoculated control in a consistent and reproducible way (Dubrovsky et al. 1994). Subsequently, it was established that this effect had a strong phytohormonal component mediated by IAA (Spaepen et al. 2014).

The study of the *Azospirillum* genome began in 2000 with the analysis of five *Azospirillum* spp. genomes using pulsed-field gel electrophoresis (Martin-Didonet et al. 2000). This biochemical characterization continued, and new plant growth-promoting mechanisms were proposed. It was found that *Azospirillum* was able to solubilize insoluble phosphates through the production of gluconic acid (Rodríguez et al. 2004). The same year, the sequence of the pRhico plasmid in *A. brasilense* Sp7 was analyzed and it was found to have an important role in plant-root interactions and bacterial viability (Vanbleu et al. 2004). In 2005, it was demonstrated that the nitric oxide produced in vitro by *A. brasilense* Sp245 was a promoter of lateral root initiation in tomato seedlings (Creus et al. 2005). Another interesting mechanism emerged in 2006. Four strains belonging to *A. lipoferum* isolated from the rice rhizosphere were able to synthesize N-acyl-homoserine lactones (AHLs), which regulate crucial functions for plant-bacteria interactions (Vial et al. 2006). A similar paper reported the production of cadaverine by *A. brasilense* Sp245 and Az39 (Perrig et al. 2007). Another study confirmed that *A. brasilense* had the capacity to produce several polyamines, such as putrescine, spermine, and spermidine, under similar culture medium conditions (Thuler et al. 2003). Supporting evidence was reported in later studies. It was reported that *A. brasilense* Az39 promoted root growth and helped mitigate osmotic stress in rice seedlings, in part due to cadaverine production (Cassán et al. 2009).

In 2010, the complete genomic structure of *Azospirillum* sp. B510 isolated from stems of rice plants was obtained (Kaneko et al. 2010). That study was the first to report on the genome structure of a member of the genus *Azospirillum*. In the same year, a new hypothesis about the action of *Azospirillum* on plants was proposed (Bashan and de-Bashan 2010). A year later, Wisniewski-Dyé et al. (2011) obtained the genome sequences of the model strains *A. brasilense* Sp245 and *A. lipoferum* 4B and analyzed the taxonomic origin of this bacterial genus. Through genome sequencing and analysis, they showed that *Azospirillum* spp. transitioned from aquatic to terrestrial environments. Most of the *Azospirillum* genes were acquired horizontally, and they encode functions that are critical for rhizosphere-plants adaptation and interaction. In 2014, the complete genome sequence of *A. brasilense* Az39 was presented (Rivera Botia et al. 2014); it is one of the strains that is most often used for agriculture in South America. One year later, the complete genome sequences of *A. brasilense* Sp7 (Kwak and Shin 2015) and *A. thiophilum* isolated from a sulfide spring (Fomenkov et al. 2016) were analyzed and annotated. More recent studies have identified the draft genome sequences of *Azospirillum* sp. B2, isolated from a raised *Sphagnum* bog (Grouzdev et al. 2018), *A. brasilense* strains Ab-V5 and Ab-V6 (Hungria et al. 2018), extensively used as biofertilizers in Brazil, and *A. brasilense* REC3 (Fontana et al. 2018), isolated from

strawberry plants in Argentina. Recently, the quorum-sensing and quorum-quenching mechanisms based on N-acyl-L-homoserine lactones in *A. brasilense* Az39 were analyzed in silico and in vitro (Gualpa et al. 2019). That study reported that although *A. brasilense* Az39 this strain is a silent bacterium unable to produce AHL signals, it can interrupt the communication between other bacteria and/or plants via its quorum-quenching activity.

Concluding remarks and perspectives

Since its re-discovery in the 1970s, *Azospirillum* has become a cornerstone in the study of PGPB. Its potential as an effective inoculant for a wide variety of crops has been recognized. Yet, the exact mode of action is still not completely understood. *Azospirillum* modes of action were initially explained by the Additive Hypothesis; 20 years later, that was replaced by the multiple mechanisms hypothesis. In this review, we proposed the Efficient Nutrients Acquisition Hypothesis, which posits that plant growth promotion occurs via two major mechanisms, biological N fixation and phytohormone production, which are effectively induced by the colonized bacteria. Thus, some of these molecules have the capacity to alter the root morphology, thereby improving mineral uptake and inducing higher yields, even if using lower doses of chemical fertilizers. The contribution of N fixation is more controversial, and its effect may be less potent than previously believed. Although mixed results have been reported for inoculation, this has not prevented numerous companies around the world from offering inoculants containing *Azospirillum*. More specifically, in South America, 10 million doses of inoculants containing *Azospirillum* were used in 2018.

The use of *Azospirillum* under field conditions has been widely shown to improve plant growth and crop productivity. Thus, the use of azospirilla inoculants for crop production should be understood as a consolidated practice, in terms of grain yield production in summer and winter cereals, as well as legume production (co-inoculation). As an improvement in the use of *Azospirillum*, co-inoculation with rhizobia has proven to be a novel technology to enhance legume performance. Part of the current challenges of azospirilla inoculants has been the need for inoculant companies to develop effective formulations that can be used for diverse applications and under different storage handling and environmental conditions. In summary, the development of alternative application systems, such as the delivery of azospirilla by foliar inoculation, is seen as a solution to overcoming the limitations of on-seed treatment. There is an urgent need to promote a regional coordinated communication program about the already measured benefits of inoculation with *Azospirillum* as a complement to current extensive and intensive crop practices. These networks should include direct users of these products as well

as other actors from rural and urban environments and local regulatory agencies.

Additionally, *Azospirillum* inoculation may serve as a valuable method for the remediation of contaminated soil and water and the revegetation and reforestation of degraded lands. Furthermore, the interaction of *Azospirillum* with green microalgae was proven to be an independent sub-field of *Azospirillum* research, presenting a new and interesting avenue to produce metabolites, such as lipids and pigments. However, this biotechnological application is yet to be tested under scale-up conditions to evaluate its real-life potential.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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