CHAPTER TWO

How the Plant Growth-Promoting Bacterium *Azospirillum* Promotes Plant Growth—A Critical Assessment

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Contents

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This review is dedicated to the memory of Dr. Wolfgang Zimmer (1958–2002) from Fraunhofer-Institute of Atmosphere Research in Garmisch-Partenkirchen, Germany. He intensively studied mechanisms of action by *Azospirillum* in the 1980s and 1990s.

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Abstract

During the last 35 years of studies of *Azospirillum*–plant interaction, over 20 proposals were suggested for the mechanism of action by which *Azospirillum* spp., the most intensively studied plant growth-promoting bacteria, enhances plant growth. The proposals include a single phytohormone activity, multiple phytohormones, nitrogen fixation, assortments of small-sized molecules and enzymes, enhanced membrane activity, proliferation of the root system, enhanced water and mineral uptake, mobilization of minerals, mitigation of environmental stressors of plants, and direct and indirect biological control of numerous phytopathogens. By volume, the largest number of published information involves hormonal activities, nitrogen fixation, and root proliferation. After analyzing the accumulated knowledge, it was concluded that this versatile genus possesses a large array of potential mechanisms by which it can effect plant growth. Consequently, this review proposes the "Multiple Mechanisms Theory," based on the assumption that there is no single mechanism involved in promotion of plant growth by *Azospirillum*, but a combination of a few or many mechanisms in each case of inoculation. These may vary according to the plant species, the *Azospirillum* strain, and environmental conditions when the interaction occurred. The effect can be cumulative, an "additive hypothesis" (proposed before), where the effects of small mechanisms operating at the same time or consecutively create a larger final effect on plant. Additionally, the observed effect on plant growth can be the result of a tandem or a cascade of mechanisms in which one mechanism stimulates another, yielding enhanced plant growth, such as the plausible relations among phytohormones, nitric oxide, membrane activities, and proliferation of roots. Finally, the growth promotion can also be a combination of unrelated mechanisms that operate under environmental or agricultural conditions needed by the crop at particular locations, such as mitigating stress (salt, drought, toxic compounds, adverse environment), and the need for biological control of or reducing pathogenic microflora.

1. Introduction

Since the rediscovery in the mid-1970s of the genus *Azospirillum* as a plant-associated bacteria by the late Johana Döbereiner and her collaborators in Brazil and its definition as a plant growth-promoting bacteria (PGPB; Döbereiner and Day, 1976), two main characteristics defined the genus; it fixes atmospheric nitrogen and produces phytohormones (Tien *et al.*, 1979). Consequently, these two features were considered, from the onset of plant–bacteria studies, as the cornerstone of the effect of this genus on plant growth and yield. Because *Azospirillum* is the most studied PGPB, excluding rhizobia, and reached commercialization in several countries, including Argentina, Mexico, India, Italy, and France (Díaz-Zorita and

Fernández-Canigia, 2009; Hartmann and Bashan, 2009), considerable knowledge has been accumulated during the last three decades, showing more and different facets of this interaction.

Despite intensive studies of the physiology and molecular biology of this genus, mostly as an easy-to-handle laboratory model of a rhizosphere bacterium, the exact mode of action of the bacteria on plants is not much clearer than it was decades ago (Bashan and Holguin, 1997; Bashan and Levanony, 1990; Bashan *et al.*, 2004). There are three facts that are beyond dispute, each with a reservation: (1) Most *Azospirillum* strains can fix nitrogen but only a fraction of it, if any at all, is transferred to the plant; (2) Many strains, but not all, produce several phytohormones *in vitro* and also a few in association with plants, but transfer of hormones is probably limited and was not always detected and only assumed to occur; (3) A general positive growth response in numerous plant species is evident in the majority of cases of inoculation, but the effect is not always apparent in terms of economic productivity (Díaz-Zorita and Fernández-Canigia, 2009; Okon and Labandera-Gonzalez, 1994). These concerns accelerate the research into alternative mechanisms.

The most apparent outcomes of most inoculations with *Azospirillum* are the major changes in plant root architecture. Inoculation can promote root elongation (Dobbelaere *et al.*, 1999; Levanony and Bashan, 1989), development of lateral and adventitious roots (Creus *et al.*, 2005; Fallik *et al.*, 1994; Molina-Favero *et al.*, 2008), root hairs (Hadas and Okon, 1987; Okon and Kapulnik, 1986), and branching of root hairs (Jain and Patriquin, 1985), some of which occurred in many plant species, consequently significantly increasing and improving their root system. It is generally accepted that these developmental responses in root morphology are triggered by phytohormones, possibly aided by their associated molecules. The fundamental question is: one hormone or several or a fine-tuned combination among several hormones?—all of which were produced by the bacterium, but mainly *in vitro*.

Inoculated plants absorbed more minerals and water, and in many cases, were more vigorous and greener, and showed enhanced plant growth. Several possible mechanisms were suggested to explain these phenomena, some with more experimental data than others. Yet, there is no definite agreement on exactly how the bacteria can effect plant growth. Is this a result of an input by the bacteria and can we manipulate it? Naturally, a multitude of proposed mechanisms claim to the lead mechanism responsible for the observed effects on plant growth, and specifically, on plant yield, that in most case, is the desired outcome and the main reason for inoculation. These questions are the driving force in *Azospirillum* research today, because if we have a clearer idea how the bacterium interacts with its host, we may envision ways to improve the interaction.

The aims of this chapter are to critically assess the large amount of knowledge on the possible plant promoting mechanisms of *Azospirillum* (Table 1, Fig. 1) and to present potential avenues for clarifications of this open question or at least some starting points for future research.

2. Major Mechanisms

2.1. Production of phytohormones

The ability to form plant hormones is a major property of many microorganisms and PGPB in general and specifically, species of *Azospirillum* that stimulate and facilitate plant growth (Tsavkelova *et al.*, 2006). This is believed to be part of the mutualistic relationships developed between plants and their associate bacteria. *Azospirillum* spp. are known for their ability to produce plant hormones, as well as polyamines and amino acids in culture media (Hartmann and Zimmer, 1994; Thuler *et al.*, 2003). Among these hormones, indoles, mainly indole-3-acetic acid (IAA; Spaepen *et al.*, 2007a), and gibberellins (GAs) of several kinds (Bottini *et al.*, 2004) may play a larger role. These phytohormones alter metabolism and morphology of plants, leading to better absorption of minerals and water, consequently larger and healthier plants. In the unicellular microalga *Chlorella vulgaris*, phytohormones lead to larger cell populations (de-Bashan *et al.*, 2008a). Thus far, hormonal effects are the mode of action for the largest volume of experimental data, and it is presented, justifiably or not, as the major (and sometimes as the sole) contribution of *Azospirillum* to plant growth.

The topic of IAA in PGPB in general and *Azospirillum* in particular and specifically the genes involved in synthesis of IAA were intensively, continuously, and excellently reviewed during the last decade (Costacurta and Vanderleyden, 1995; Dobbelaere *et al.*, 2003; Patten and Glick, 1996; Spaepen *et al.*, 2007a,b; Steenhoudt and Vanderleyden, 2000; Vande Brock and Vanderleyden, 1995), and therefore, this review will present only a few key points for and against their proposal.

2.1.1. Indole-3-acetic acid

IAA is a heterocyclic compound containing a carboxymethyl group (acetic acid) that belongs to the auxin phytohormone family. It is the best characterized and the most studied phytohormone and involved in numerous mechanisms in plant physiology. Auxins are responsible for division, extension, and differentiation of plant cells and tissues. Phytohormones of this group increase the rate of xylem and root formation; control processes of vegetative growth, tropism, florescence, and fructification of plants; and also affect photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to biotic stress factors. In microorganisms, in general, the

Table 1 General evaluation of proposals for mode of action on plants by *Azospirillum*

Proposal	Year proposed and significance with current knowledge[a]	Description	Evidence for	Arguments against and/or lack of evidence for	References (examples)[b]
Phytohormones	1979-present (+++)	*Azospirillum* can produce *in vitro* several phytohormones. External application of synthetic hormones or hormones purified from bacterial culture imitated the positive effects of *Azospirillum* on root development			Costacurta and Vanderleyden (1995), Spaepen *et al.* (2007a), Steenhoudt and Vanderleyden (2000), Vande Brock and Vanderleyden (1995)
IAA	1979-present (+++)	IAA is produced by the bacterium *in vitro* in large quantities and is attributed to affect numerous alterations in plant functions yielding eventually growth promotion. Significant knowledge about IAA metabolism and molecular	1. Most strains of *Azospirillum* produce IAA *in vitro* 2. In several cases, IAA-attenuated mutants were ineffective compared to their wild-type parental strains 3. Application of IAA mimics *Azospirillum* inoculation on	1. Only several cases of direct involvement of IAA in growth promotion of plants are known 2. There are no IAA-deficient mutants 3. Most demonstrated cases are indirect or circumstantial 4. IAA is produced by plant cells and IAA detected in the plants were only indirectly induced, but not	Baca *et al.* (1994), Barbieri and Galli (1993), Bothe *et al.* (1992), de-Bashan *et al.* (2008a), Dobbelaere *et al.* (1999), El-Khawas and Adachi (1999), Fallik *et al.* (1989), Gonzalez and Bashan (2000), Hartmann *et al.* (1983), Malhotra and Srivastava (2008), Molla *et al.* (2001), Omay *et al.* (1993),

(*continued*)

Table 1 (*continued*)

Proposal	Year proposed and significance with current knowledge[a]	Description	Evidence for	Arguments against and/or lack of evidence for	References (examples)[b]
		mechanism in the bacterium is known	root morphology and growth promotion of plants and single cell algae 4. IAA is involved in numerous functions in the plant cells and therefore might be a part of a cascade employing other mechanisms 5. Elevated IAA was detected in inoculated plants 6. IAA overproducing mutants showed stronger effect on plants	directly produced by the bacterium 5. Direct transfer of bacterial IAA into plant cells and its functional consequences are still lacking 6. Production of bacterial IAA in the plant was not demonstrated 7. Cases of no evidence of correlation between capacity of IAA biosynthesis and root growth promotion 8. Is additional IAA *in planta* better? 9. Studies showing that *Azospirillum*-IAA biosynthesis alone cannot account for the overall growth promotion observed	Remans *et al.* (2008), Spaepen *et al.* (2007b, 2008), Zimmer *et al.* (1991)

Gibberellins (GA)	1989-present (++)	GA affecting plant development in similar manner like auxins with several differences. GA promotes cell division and elongation and are involved in breaking dormancy	1. GAs are synthesized and metabolized by *Azospirillum in vitro* 2. GA are produced *in planta* by *Azospirillum* 3. Inoculation of GA-deficient mutant dwarf rice mutants with *Azospirillum*-GA producer reversed dwarfism	Insufficient evidence for involvement of bacterial GA in promoting growth	Bottini *et al.* (1989, 2004), Cassan *et al.* (2009a,b), Fulchieri *et al.* (1993), Piccoli and Bottini (1994a,b), Piccoli *et al.* (1997, 1999), Perrig *et al.* (2007)
Cytokinins	1979 (UN)	Cytokinins are involved in cell enlargement and division, shoot and root morphogenesis and senescence	Cytokinins were produced by *Azospirillum in vitro*	No direct evidence in plants. Insufficient data.	Cacciari *et al.* (1989), Horemans *et al.* (1986), Strzelczyk *et al.* (1994), Tien *et al.* (1979)
Abscisic acid (ABA)	2007-present (UN)	ABA is involved in response to environmental stress such as heat, water, and salt	1. This compound was found *in vitro* in several strains 2. Interaction between GA and ABA in water-stress mitigation of plants	Insufficient evidence for involvement of bacterial ABA in growth promotion	Cohen *et al.* (2008, 2009), Perrig *et al.* (2007)

(*continued*)

Table 1 (*continued*)

Proposal	Year proposed and significance with current knowledge[a]	Description	Evidence for	Arguments against and/or lack of evidence for	References (examples)[b]
Ethylene	2006-present (+)	Ethylene plays a role in breaking dormancy of seeds. Its main effect is in senescence of the plant	1. Ethylene was found in culture filtrate of *A. brasilense* 2. Growth promotion was associated with low ethylene levels in tomato 3. Insertion gene of ACC-deaminase in *Azospirillum* improve plant growth	Too few cases of ethylene involvement in *Azospirillum*, to compare with other PGPB, where it is a major mechanism	Holguin and Glick (2001, 2003), Perrig *et al.* (2007), Prigent-Combaret *et al.* (2008), Ribaudo *et al.* (2006)
Polyamines: cadaverine, putrescine, spermine, and spermidine	2003-present (UK)	Unclear function. Can act as growth regulating compounds	1. These compounds were found *in vitro* 2. Application of cadaverine mitigated osmotic stress in rice	Limited data	Cassan *et al.* (2009a), Perrig *et al.* (2007), Thuler *et al.* (2003)
Enhanced root growth combined with enhanced	1979-present (+++)	Inoculation caused a more developed root system that allows better uptake	1. Enhanced root system is the most common phenotypical effect of inoculation with	1. Most data is descriptive and does not show whether the improvements are the cause or the	Bashan *et al.* (1990), Jain and Patriquin (1984), Kapulnik *et al.* (1981, 1985b), Lin *et al.* (1983),

mineral and water uptake		of water and minerals	*Azospirillum* in most plant species 2. Enhanced mineral and water uptake by plants follow inoculation	results of other mechanisms 2. The wide range of enzymes related to these phenomena was only slightly studied 3. Despite the large volume of information, relatively few strains were evaluated	Morgenstern and Okon (1987), Murty and Ladha (1988), Ogut and Er (2006), Sarig *et al.* (1988, 1992)
Nitrogen fixation	1975-present (++)	Nitrogen fixation is a common feature of most *Azospirillum* species.	1. Following inoculation significant increase in total N in shoots and grain 2. Many greenhouse and field experiments indicate some contribution of fixed N in the plant 3. Inoculation commonly reduced the level of N fertilization needed for many plant species	Many studies showed little or minimal contribution of fixed nitrogen in the plant. Some systems showed none	Baldani and Baldani (2005), Bashan *et al.* (1989b, 2004), Choudhury and Kennedy (2004), Christiansen-Weniger (1992), Garcia de Salamone *et al.* (1997), Katupitiya *et al.* (1995a,b), Kennedy and Islam (2001), Kennedy *et al.* (2004), Mirza *et al.* (2000), Rodrigues *et al.* (2008), Saubidet and Barneix (1998), Sriskandarajah *et al.*

(*continued*)

Table 1 (*continued*)

Proposal	Year proposed and significance with current knowledge[a]	Description	Evidence for	Arguments against and/or lack of evidence for	References (examples)[b]
			4. Enhanced nitrogenase activity in inoculated plants 5. The contribution of fixed N was apparent in many para-nodule systems		(1993), Van Dommelen *et al.* (2009)
Nitric oxide (NO)	2005-present (+)	NO is a free radical which participates in metabolic, signaling, defense, and developmental pathways in plants	1. *Azospirillum* can produce NO *in vitro* by different pathways 2. NO can modify root architecture	Limited data	Creus *et al.* (2005), Molina-Favero *et al.* (2007, 2008)
Nitrite production	1992 (UN)	*Azospirillum* can produce nitrite as part of its normal metabolism	1. Nitrite participated in plant growth promotion 2. Nitrite can cause sharp decrease in formation of lateral roots	Limited data	Bothe *et al.* (1992), Zimmer *et al.* (1988)

Nitrate reductase (NR)	1987 (UN)	An explanation for accumulating nitrogen following *Azospirillum* inoculation	1. NR activity of wheat leaves was decreased by inoculation with some *Azospirillum* strains 2. Increase in nitrate assimilation	Limited data	Boddey and Döbereiner (1988), Ferreira *et al.* (1987)
Phosphate solubilization and mineral weathering	1998-present (UN)	Solubilization of several rock minerals especially P making them available for the plant	Several strains can solubilize nonsoluble P and other minerals from rocks and stones	Limited data	Carrillo *et al.* (2002), Chang and Li (1998), Kamnev *et al.* (1999a, 2002b), Puente *et al.* (2004a,b, 2006), Rodriguez *et al.* (2004), Seshadri *et al.* (2000)
Effect on plant membranes and enhanced proton extrusion	1989-present (UN)	Inoculation induces root cell membranes to release protons	1. Short exposure of roots to *A. brasilense* significantly enhanced the proton efflux of the root 2. Inoculation significantly reduced the membrane potential in every root part	1. Signal molecules in bacteria that might affect membranes were not identified 2. Mobilization of ions via the affected membranes was not studied	Alen'kina *et al.* (2006), Amooaghaie *et al.* (2002), Antonyuk *et al.* (1993, 1995), Bashan (1990, 1991), Bashan and Levanony (1991), Bashan *et al.* (1989a, 1992), Carrillo *et al.* (2002), Nikitina *et al.* (2004)

(*continued*)

Table 1 (*continued*)

Proposal	Year proposed and significance with current knowledge[a]	Description	Evidence for	Arguments against and/or lack of evidence for	References (examples)[b]
			3. Inoculation changed the phospholipid content in plant membranes 4. *Azospirillum* produces lectins. Some can cause change in growing cells mitosis 5. Wheat germ agglutinin from plants enhanced several metabolic pathways in *Azospirillum*		
Mitigation of environmental stress	1988-present	Best effects of inoculation occurred when plants are grown under suboptimal conditions			Bashan and Holguin (1997), Bashan and Levanony (1990), Bashan *et al.* (2004)
Salinity	1997-present (++)	Inoculated plant under saline condition grow better	1. Inoculation improved germination, plant development	Missing information on: 1. Relation between salt tolerance of the bacterium and those of the plant	Bacilio *et al.* (2004), Barassi *et al.* (2006), Creus *et al.* (1997), Hamdia and

			2. Increases in content of water, chlorophyll, essential minerals, proteins, amino acids, enhanced uptake of K and Ca, NR, and nitrogenase 3. Restricted Na uptake	2. What are the physiological mechanisms involved? 3. How do the bacteria induce these effects?	El-Komy (1997), Hamdia *et al.* (2004)
Drought	1988-present (++)	Inoculated plant under drought or osmotic stress are growing better	Inoculation improved plant growth, reduce grain loss, improve water content, increased turgor pressure, positive effect on cell wall elasticity, higher Mg, K, and Ca in grains, and improve fatty acid distribution profile	Too little data about the physiological mechanisms involved	Alvarez *et al.* (1996), Creus *et al.* (1998, 2004), El-Komy *et al.* (2003), Pereyra *et al.* (2006), Sarig *et al.* (1990)
Metal toxicity	2000-present (+)	Reduction in toxicity to plants	1. The bacterium tolerates medium levels of metals	Inoculation does not provide full protection against metal toxicity	Belimov and Dietz (2000), Belimov *et al.* (2004), Kamnev *et al.*

(*continued*)

Table 1 (*continued*)

Proposal	Year proposed and significance with current knowledge[a]	Description	Evidence for	Arguments against and/or lack of evidence for	References (examples)[b]
			2. Inoculation allows plants to grow in metal contaminated soils and in mine tailings		(2005, 2007), Lyubun *et al.* (2006)
Humic acid toxicity	2003 (UN)	Reduction in toxicity to plants	1. Improved germination and plant growth at elevated humic acids 2. Consumption of humic acid by the bacterium	Inoculation does not provide full protection against toxicity	Bacilio *et al.* (2003)
pH and tryptophan in aquatic environment	2005-present (+)	Inoculation allows microalgae to grow under unfavorable aquatic conditions	Inoculated microalgae can grow in high pH and toxic levels of tryptophan	Unknown	de-Bashan and Bashan (2008), de-Bashan *et al.* (2005)
High light intensity	2006-present	Inoculation allows plants to grow under high light intensity	1. Inoculated wheat plants produced photoprotective photosynthetic pigments	Unknown	Bashan *et al.* (2006), de-Bashan *et al.* (2008b)

			2. Inoculation allowed microalgae to grow under extreme light intensities		
Herbicide	1997 (UN)	Reduction in toxicity to plants	Cotton plants were partially protected from the herbicide 2-4-D	Unknown	Feng and Kennedy (1997)
Biological control of pathogens	1990-present (++)	Indirect effects on plant growth reducing the deleterious effects of pathogens	1. *Azospirillum* produces a variety of inhibitory substances 2. Inhibits germination and development of parasitic weeds 3. Can compete with phytopathogens 4. Inhibits development of microfauna and insects 5. Inhibits foliar bacterial diseases and soil-borne fungal pathogens	1. Most studies are descriptive 2. Almost all mechanisms were not studied or are in initial stage	Bashan and de-Bashan (2002a), Dadon *et al.* (2004), Gonçalves and de Oliveira (1998), Kavitha *et al.* (2003), Khan and Kounsar (2000), Romero *et al.* (2003), Sudhakar *et al.* (2000)

(*continued*)

Table 1 (*continued*)

Proposal	Year proposed and significance with current knowledge[a]	Description	Evidence for	Arguments against and/or lack of evidence for	References (examples)[b]
Additive hypothesis	1990-present	The effects of small mechanisms operating at the same time or consecutively create a larger final effect on plant			Bashan and Dubrovsky (1996), Bashan and Levanony (1990)
Multiple mechanisms	2010	A combination of a few or many mechanisms in each case of inoculation			This essay

[a] +++, possibly major mechanism; ++, possibly moderate; +, possibly minor; UN, unknown.
[b] More comprehensive literature are listed in the text.

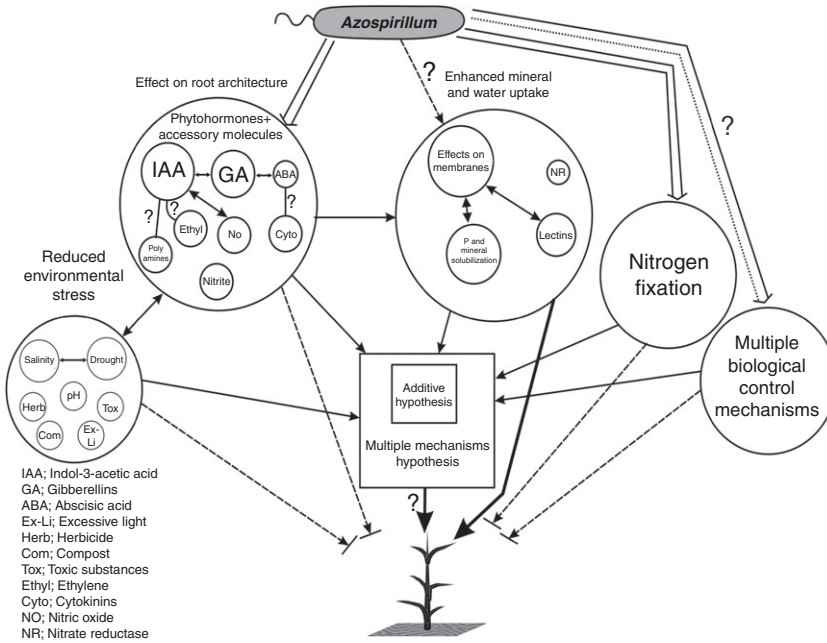

Figure 1 Mechanisms by which *Azospirillum* spp. may enhance plant growth and their possible interactions grouped as biological processes. Circles represent processes containing experimental data. Squares represent theories. Size of a circle represents its relative importance according to current data. Solid arrow: mechanism(s) that can fully create the observed growth promotion; dash arrow: mechanism(s) that can only partially explain the observed growth promotion. Simple arrows: proven interactions among different mechanisms; double-line arrow: direct production of molecules or processes by the bacterium cell; ?: unproven as yet, or partially proven pathway.

three known pathways of IAA biosynthesis are related to tryptophan metabolism (amino acid frequently found in plant exudates; Costacurta and Vanderleyden, 1995; Patten and Glick, 1996). Omission of tryptophan from the culture medium decreases the level of IAA synthesis by the culture's microorganisms. Addition of exogenous tryptophan (or, more rarely, tryptamine) may augment auxin biosynthesis by an order of magnitude or even greater. The known routes of IAA biosynthesis includes: (1) IAA formation via indole-3-pyruvic acid (IPyA) and indole-3-acetaldehyde; (2) Conversion of tryptophan into indole-3-acetaldoxyme and indole-3-acetonitrile (IAN); and (3) IAA biosynthesis via indole-3-acetamide formation (IAM; Zakharova *et al.*, 1999). It has been reported that a tryptophan-independent pathway, more common in plants, was also found in azospirilla (Carreño-Lopez *et al.*, 2000; Prinsen *et al.*, 1993). However, the contribution of this pathway to IAA biosynthesis is questionable, and the mechanisms are largely unknown.

Involvement of tryptophan in IAA production by *Azospirillum* has been known for a long time (Reynders and Vlassak, 1979). A key gene *ipdC* encodes for indole pyruvate decarboxylase. This is a key enzyme in the IAA synthesis pathway by *A. brasilense* that mediates conversion of IPyA into indole-3-acetaldehyde; its presence presented conclusive evidence for the IPyA pathway in this bacterium (Costacurta *et al.*, 1994). Zimmer *et al.* (1998) isolated the *ipdC* gene from strain Sp7 of *A. brasilense* and showed tryptophan-dependent stimulation of gene expression in this bacterium. These two findings were later confirmed by IAA production by several strains of *Azospirillum* where production depended on the type of culture media and availability of tryptophan as a precursor (e.g., El-Khawas and Adachi, 1999; Malhotra and Srivastava, 2006, 2008). The pH of the culture medium has a significant effect on the amount of IAA produced (Ona *et al.*, 2003). Release of large amounts of IAA by *Azospirillum* spp. cultures is probably controlled by the stationary phase of the bacteria cells after depletion of the carbon source in the medium used in batch culture. Depletion of the carbon source reduces growth (Ona *et al.*, 2003, 2005). Assessment of possible precursors (indole, anthranilic acid, and tryptophan) for IAA formation in *A. brasilense* Sp245 revealed a high motive force for tryptophan synthesis from chorismic acid and for IAA synthesis from tryptophan (Zakharova *et al.*, 1999). Vitamins may also play a role in the regulation of IAA synthesis in *A. brasilense*. Very low levels of B vitamins, especially pyridoxine and nicotinic acid, increased production of IAA in *A. brasilense* (Zakharova *et al.*, 2000).

To demonstrate direct involvement of IAA produced by *A. brasilense* on plant growth, it would be preferable, if not essential, to use IAA-deficient mutants. It is relatively straightforward to obtain IAA overproducing mutants (Hartmann *et al.*, 1983) but, so far, almost impossible to obtain IAA-deficient mutants. This occurs because of the different pathways that *Azospirillum* spp. has to produce IAA (Spaepen *et al.*, 2007a; Zakharova *et al.*, 1999). For example, in most mutants, the unstable indole pyruvic acid spontaneously breaks down and produces some IAA (Steenhoudt and Vanderleyden, 2000). These IAA-attenuated mutants produce 0.2–10% of the level of IAA produced by the wild type, sometimes even more. Quite a few of these strains were found or constructed and used. A strain of *A. irakense* released about 10 times less IAA into the medium than *A. brasilense* Sp7 (Zimmer *et al.*, 1991). Two mutants of *A. brasilense* produced 2–5% of the IAA produced by the parental strains (Prinsen *et al.*, 1993, Vande Broek *et al.*, 1999). Mutants of *A. brasilense* and *A. lipoferum* that were modified to include the *gfp* (green fluorescent protein) gene produced less than 0.25% IAA of their parental strains (Bacilio *et al.*, 2004; Rodriguez *et al.*, 2006) and mutant of *A. brasilense* Sp6, carrying another Tn5 insertion in the *ipdC* gene, produced less than half the IAA of its parental strain (Barbieri and Galli, 1993). Recently, an *ipdC*-knockout mutant was found to produce

only 10% of the wild-type IAA production level (Spaepen *et al.*, 2007b). Furthermore, when the endogenous promoter of the *ipdC* gene was replaced by either a constitutive or a plant-inducible promoter and both constructs were introduced into the wild-type strain, the introduction of these recombinant *ipdC* constructs improved the growth-promoting effect of *A. brasilense* (Spaepen *et al.*, 2008).

IAA is produced during all stages of culture growth and well after the stationary phase (Malhotra and Srivastava, 2009). This feature makes the bacterium especially qualified for plant growth promotion when the effect last weeks or months after inoculation. Consequently, IAA production by *Azospirillum* sp. was proposed to play a major role in growth promotion and even more auxin-type molecules were detected in *Azospirillum*, such as indole butyric acid (IBA; Fallik *et al.*, 1989), indole lactic acid (Crozier *et al.*, 1988), indole acetamide (Hartmann *et al.*, 1983), indole acetaldehyde (Costacurta *et al.*, 1994), indole ethanol and indole methanol (Crozier *et al.*, 1988), and phenyl acetic acid (Somers *et al.*, 2005). Nonetheless, when compared to the large base of knowledge on IAA production by the bacterium cell, a far smaller volume of indirect and direct evidence regarding the effect of IAA of bacterial origin in plants has been published.

In general, morphological changes in roots, following *Azospirillum* inoculation, were mimicked by applying a combination of plant growth substances, which point to involvement of an auxin produced by *Azospirillum* for root proliferation and consequent plant growth promotion (for reviews, see Bashan and Holguin, 1997; Bashan *et al.*, 2004). Specific evidence for the involvement of auxins in promoting plant growth includes elevated IAA and IBA in *Azospirillum*-inoculated maize plants (Fallik *et al.*, 1989). Addition of filter-sterilized culture supernatants to rice roots grown in hydroponic tanks increased root elongation, root surface area, root dry matter, and development of lateral roots and root hairs, compared with untreated roots. Higher concentrations of the supernatant strongly inhibited root elongation, lateral root development, and caused nodule-like tumors on the roots (El-Khawas and Adachi, 1999). Similarly, a cell-free supernatant of *A. brasilense* Cd applied to soybean plants induced many roots and increased root length (Molla *et al.*, 2001). Inoculation of wheat with wild strains of *A. brasilense* Sp245 and Sp7 led to an exceptional decrease in root length and increase in root hair formation, as is common with such inoculations. The effect on root morphology was further enhanced by adding tryptophan; this could be mimicked by replacing *Azospirillum* cells with IAA (Dobbelaere *et al.*, 1999). Exogenous application of IAA to bean roots resembled responses of these plants to inoculation with *Azospirillum* (Remans *et al.*, 2008). Similarly, application of IAA directly to growing cells of the freshwater microalgae *C. vulgaris* mimicked cell proliferation induced by *Azospirillum* (Gonzalez and Bashan, 2000). More direct evidence for the importance of IAA was provided when several IAA-attenuated mutants

were compared with their parental wild types for their effect on the growth of this microalga, when only the wild types were capable of promoting growth. Yet, adding culture filtrate of these wild types to cultures of IAA-attenuated mutants, incapable of inducing microlagal growth, restored their effect on microalgal growth (de-Bashan *et al.*, 2008a). A mutant of *A. brasilense* with low production of phytohormones, but high N_2-fixation activity, did not enhance root growth over uninoculated controls. In contrast, a mutant with increased phytohormone production significantly affected root morphology. In general, increased plant biomass and N_2-fixation were recorded in strains having increased production of indole compounds (Kundu *et al.*, 1997). Further study of the contribution of auxin biosynthesis by *A. brasilense* in altering root morphology and root proliferation showed that inoculation of wheat seedlings with an *A. brasilense* Sp245 strain, carrying a mutation in the *ipdC* gene, which did not cause shorter roots or stimulate root hair formation, in contrast to inoculation with the wild type (Dobbelaere *et al.*, 1999). The insertion of the heterologous IAM pathway, consisting of the *iaa*M and *iaa*H genes into *A. brasilense* SM increased IAA levels by threefold and the engineered strain showed a superior effect on the lateral branching of sorghum roots, as well as its dry weight when compared with the wild-type strain (Malhotra and Srivastava, 2006).

Several studies showed no evidence of correlation between the capacity for IAA synthesis by *A. brasilense* and the effects on observed root growth promotion (Bothe *et al.*, 1992; Harari *et al.*, 1988; Kapulnik *et al.*, 1985a). Additionally, several studies showed that *Azospirillum*-IAA biosynthesis alone cannot account for the overall growth stimulatory effect observed (for a review, see Spaepen *et al.*, 2007a).

In summary, although evidence of IAA production in *Azospirillum* spp. is the most comprehensive and documented from all hormones or suggested mechanisms, the direct evidence of involvement of this hormone as the *sole* mechanism by which the bacteria affect plant growth is, in our opinion, unproven, although it is very likely that IAA is involved in many of the interactions of this genus. It is feasible though to consider a hormonal effect in very early stages of germination. Most stains of *Azospirillum*, when fermented as an inoculant, are capable of producing IAA and other growth regulators at a concentration sufficient to produce morphological and physiological change in young seed tissues. Such initial "phytohormonal shock" would be the first contact between the bacterial inoculant and the seed and would not necessarily depend on the presence of bacteria. However, the presence of live bacteria may contribute to *in situ* phytohormone production over a longer term. According to this concept, bacterial phytostimulation would be crucial in early developmental stages (germination and initial seedling growth) and will be complementary to other mechanisms operating at later stages of *Azospirillum* interaction with plants (Cassan *et al.*, 2009b), as summarized in Section 3.6.

2.1.2. Gibberellins and abscisic acid

GAs, diterpenoid acids that are synthesized by the terpenoid pathway, are hormones (over 120 types have been found in plants, fungi, and bacteria) that control growth and a wide variety of other plant developmental processes similar to auxins. Primarily, they promote cell division and elongation, but without inhibitory effects presented by some auxins. Additionally, GAs are involved in the natural process of breaking dormancy during seed germination. GAs in the seed embryo signal starch hydrolysis by inducing the synthesis of the enzyme α-amylase in the aleurone cells. This enzyme hydrolyzes starch into glucose; the glucose is used for energy by the seed embryo. GAs cause higher levels of transcription of the gene coding for the α-amylase enzyme to stimulate the enzyme synthesis (Richards *et al.*, 2001). Despite this major role and the fact that *A. brasilense* is known to enhance germination of wheat and soybean seeds (Bacilio *et al.*, 2003; Cassan *et al.*, 2009b), GAs, so far, were not directly linked to this phenomenon. It was only shown that improved seed germination coincides with high GA production in cultures by this bacterium (Cassan *et al.*, 2009b).

Azospirillum has the capacity to synthesize and metabolize GAs *in vitro* (Bottini *et al.*, 1989; Piccoli and Bottini, 1994a,b; Piccoli *et al.*, 1996, 1997) and *in planta* (Bottini *et al.*, 2004; Cassan *et al.*, 2001a,b, and references cited therein). A growth promotion effect of *Azospirillum* spp. on plants has been suggested to be partially caused by the production of GAs by the bacterium as has occurred with other PGPB (for a review, see Bottini *et al.*, 2004). Several studies support this proposal. When a GA-producing strain of *A. lipoferum* was cultured in the presence of glucosyl ester or glucoside of GA A_{20}, both conjugates were hydrolyzed. These *in vitro* results support the hypothesis that growth promotion in plants induced by inoculation with *Azospirillum* results from a combination of GA production and GA-glucoside/glucosyl ester deconjugation by the bacterium (Piccoli *et al.*, 1997). The effect of water potential or concentration of O_2 on growth and GA A_3 (the main GA identified in *Azospirillum*) production in *A. lipoferum* showed that this GA produced by each culture was reduced severely at high water potentials or low O_2 concentrations. At the highest water potential concentration, GA A_3 was reduced by ~50%, despite a 90% reduction in cell numbers. This indicates an increase in the amount of GA A_3 produced per cell with increasing water potential (Piccoli *et al.*, 1999). Involvement of GA A_3 produced by *Azospirillum* spp. in promoting growth of maize was also suggested (Lucangeli and Bottini, 1997). *A. brasilense* Cd and *A. lipoferum* USA 5b promoted elongation of root sheaths with two single genes in GA-deficient dwarf rice mutants, dy and dx, when the inoculated seedlings were supplied with [17, 17-$^2H_2$] GA A_{20}-glucosyl ester. This growth resulted from GA metabolism by the bacteria in the dx mutant and by the rice plant

and microorganism in the dy mutant. In the dy mutant, inoculation by both bacterial strains reversed dwarfism in seedlings incubated with [17, 17-$^2H_2$] GA A_{20}, forming [17, 17-$^2H_2$] GA A_1. It is possible that the bacterial enzyme responsible for these phenomena is 2-oxoglutarate-dependent dioxygenase, similar to those of plants (Cassan *et al.*, 2009a,b).

Initial studies on the effect of *Azospirillum* spp. on plants linked GAs and bacteria-produced abscisic acid (ABA), a common isoprenoid phytohormone usually synthesized in all plant parts. ABA is ubiquitous and produced by higher plants, algae, and fungi (Zeevaart, 1999) and as a by-product of chemically defined cultures of *A. brasilense* Sp245 (Cohen *et al.*, 2008). ABA originated from its role in the abscission of leaves of only a few plant species but its main role in plants is as a response phytohormone to environmental stress, such as decreased soil water potential and heat, water, and salt stresses. ABA produced in roots is then translocated by transpiration in the xylem to the leaves, where it rapidly alters the osmotic potential of stomata guard cells, causing them to shrink and stomata to close. The ABA-induced closure of stomata reduces transpiration, preventing further water loss in times of low water availability (Bartels and Sunkar, 2005). In this stress mitigation process, ABA–GAs were investigated with *Azospirillum* inoculation even though ABA and GAs have antagonistic roles in many processes of plant growth (Achard *et al.*, 2006; Nemhauser *et al.*, 2006).

The effects of *A. lipoferum* in maize plants, in which ABA and GA synthesis were diminished by inhibitors of their own biosynthetic pathways (ABA by fluridone and GA by Ca-prohexadione) and subjected or not to drought stress, were measured. Application of fluridone diminished growth of well-watered plants similar to the effect of drought and *A. lipoferum* inoculation completely reversed this effect. The relative water content of the fluridone-treated and drought-stressed plants was significantly lower, and this effect was completely neutralized by *A. lipoferum*. The results suggest that ABA produced by the bacterium may account, at least partially, for the amelioration of growth parameters in drought-stressed and fluridone-treated plants. Similarly, growth was diminished in plants subjected to drought and treated with Ca-prohexadione, alone or combined with fluridone, even though ABA levels were higher. The results suggest that ABA and GAs participate in alleviating water stress of plants by the presence of *A. lipoferum* (Cohen *et al.*, 2009).

So far, the results indicate that, among the mechanisms involved in water-stress alleviation of plants by *Azospirillum*, is the production of stress-type hormones such as ABA (Cohen *et al.*, 2008) along with growth promoters, such as auxins (Costacurta and Vanderleyden, 1995) and GAs (Bottini *et al.*, 2004). Similar to the studies on IAA, there is far more information about GA metabolism in the bacterium that the effect of bacterial-produced GA in plants where the information about the involvement of ABA is still at an embryonic stage.

2.1.3. Polyamines

The newest compound involved in promoting growth by *Azospirillum* spp. is the polyamine cadaverine synthesized from lysine. Polyamines are low-molecular-weight organic compounds having two or more primary amino groups ($-NH_2$). Polyamines are known to be synthesized in cells via highly regulated pathways, yet, their actual function is not entirely clear. If cellular polyamine synthesis is inhibited, usually cell growth is stopped or severely inhibited. Application of exogenous polyamines restores the growth of these cells. Most eukaryotic cells have a polyamine transporter system on their cell membranes that facilitates internalization of exogenous polyamines. Polyamines serve as growth regulating compounds (Kuznetsov *et al.*, 2006); among them, cadaverine has been correlated with root growth promotion in pine and soybean (Gamarnik and Frydman, 1991; Niemi *et al.*, 2001), response to osmotic stress in turnip (Aziz *et al.*, 1997), and controlling stomata activity in *Vicia faba* beans (Liu *et al.*, 2000). *A. brasilense* strain Az39, which is a widely used as a wheat and maize inoculant in Argentina, is known to produce polyamines such as spermidine and spermine (Perrig *et al.*, 2007), and putrescine (Thuler *et al.*, 2003) in culture, and also produce cadaverine in chemically defined medium supplemented with the precursor L-lysine and in rice plants inoculated with this strain. Application of cadaverine mitigated osmotic stress in rice seedlings, based on improved water status and decreased production of ABA in inoculated seedlings (Cassan *et al.*, 2009a). Cadaverine was proposed as a contributing factor to the whole plant response to *Azospirillum* inoculation, summarized in Section 3.6 (Bashan *et al.*, 2004).

2.1.4. Cytokinins

Cytokinins are a class of purine-type phytohormones that promote cell division, shoot and root morphogenesis, chloroplast maturation, cell enlargement, auxiliary bud release, and senescence. The ratio of auxin to cytokinin is crucial during cell division and differentiation of plant tissues. Auxin is known to regulate the biosynthesis of cytokinin. The adenine-type cytokinins represented by kinetin, zeatin, and 6-benzylaminopurine occur in plants.

Cytokinins are produced in defined culture medium by many rhizosphere bacteria (Barea *et al.*, 1976), including *Azospirillum* (Cacciari *et al.*, 1989; Horemans *et al.*, 1986; Strzelczyk *et al.*, 1994; Tien *et al.*, 1979). Cytokinins from bacteria might affect plant growth positively or negatively. Apart from initial results of plants inoculated with *Azospirillum*, it is questionable if cytokinins, on their own, modified the root morphology observed in many *Azospirillum* inoculation models or if it is the levels of combination with auxin and GAs that induced the observed effect. It is hypothesized (F. Cassán, personal communication) that the contribution of

cytokinins is of some, yet undefined, importance when *Azospirillum* is combined with *Bradyrhizobium* for inoculation of soybeans. Recently, nod factors in soybean were shown as not essential for nodulation and that some strains of *Bradyrhizobium* use purines (cytokinins) as an alternative option for nodulation (Giraud *et al.*, 2007). *Azospirillum* as a potential producer of cytokinins might support this type of nodulation. It is commonly observed that inoculation with *Azospirillum* increased nodulation (Schmidt *et al.*, 1988; Yahalom *et al.*, 1990). Yet, this hypothesis is still an open proposal for future research.

2.1.5. Ethylene

During most phases of plant growth, ethylene production is minimal. Ethylene plays a major role in germination by breaking the dormancy of seeds; however, a high level of ethylene concentration inhibits subsequent root elongation. High levels of ethylene may be synthesized as a response to biological or environmental stresses, causing wilting and senescence (Glick *et al.*, 1999). Controlling ethylene levels, often by lowering them, prevents significant economic losses in agriculture. One of the precursors of ethylene synthesis is the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. ACC-deaminase is a key enzyme, commonly found in many soil microorganisms and PGPBs and capable of degrading ACC. Thus, lowering ethylene levels in plants can be considered as having potential for promoting growth (Glick *et al.*, 1999). Wild strains of *Azospirillum* spp. do not have ACC-deaminase; nevertheless, some strains can produce ethylene (Perrig *et al.*, 2007). A single exception to this role is *A. lipoferum* strain 4B that possesses the ACC-deaminase structural (*acdS*) gene (Prigent-Combaret *et al.*, 2008). This gene of the PGPB *Enterobacter cloacae* UW4 was inserted in *A. brasilense* Cd and Sp245. Roots of canola and tomato seedlings, plants sensitive to ethylene, were significantly longer in plants inoculated with the *A. brasilense* transformants than plants inoculated with nontransformed strains of the same bacterium (Holguin and Glick, 2001). In a further study, they speculated that a construct with the ACC-deaminase gene under control of a constitutive promoter weaker than the *lac* promoter, might impose less metabolic load on *Azospirillum*. The *acdS* gene was cloned under the control of a tetracycline-resistance gene promoter: *A. brasilense* Cd transformants holding *acdS* fused to the Tetr gene promoter showed lower ACC-deaminase activity than transformants with *acdS* controlled by the *lac* promoter. However, *acdS* controlled by the Tetr gene promoter exerted less metabolic load on *A. brasilense* Cd transformants than *acdS* controlled by the *lac* gene, resulting in increased IAA synthesis, growth rate, and survival of tomato leaf surfaces and ability to promote growth of seedlings (Holguin and Glick, 2003). A proposal that growth promotion triggered by inoculation with *A. brasilense* involves a signaling pathway that has ethylene as a central, positive regulator was published. The evidence is

based on higher levels of IAA and ethylene in inoculated plants. Exogenously supplied ethylene mimicked the effect of inoculation, and the addition of an inhibitor of its synthesis or of its physiological activity completely blocked promotion of growth by *A. brasilense* (Ribaudo *et al.*, 2006). Taken together, all of this may show that the involvement of ethylene in promoting growth by *Azospirillum* is probably small.

2.2. Nitrogen fixation

Since nitrogen fixation was the original proposed major mechanism by which *Azospirillum* affected plant growth (Okon *et al.*, 1983), considerable information has been published on this mechanism (for reviews, see Baldani and Baldani, 2005; Bashan and Holguin, 1997; Bashan and Levanony, 1990; Bashan *et al.*, 2004; Choudhury and Kennedy, 2004; Kennedy and Islam, 2001; Kennedy *et al.*, 2004, and references therein). The reason is that, following inoculation, there is a significant increase in the total N in shoots and grains of inoculated plants (Kapulnik *et al.*, 1981 and references in the above reviews). Incorporation of atmospheric nitrogen into the host plant by *Azospirillum* was evaluated initially by the acetylene reduction assay. However, conclusive proof that plants derive some of their N from the atmosphere came from the use of isotopic $^{15}N_2$ and ^{15}N-dilution techniques. The original seven species of this genus are diazotrophs (Bashan *et al.*, 2004). Most new species, but not all, are defined as nitrogen-fixers, either as free-living bacteria or in association with plants and participate in several transformations in the nitrogen cycle (Doroshenko *et al.*, 2007; Eckert *et al.*, 2001; Mehnaz *et al.*, 2007a,b; Peng *et al.*, 2006).

Subsequently, a very large volume of information on nitrogen-fixation mechanism in the association was published (for a review, see above). Taken together, the evidence collected during the last three decades concerning this mechanism has generated a substantial controversy. On one hand stands the numerous greenhouse and field experiments that repeatedly demonstrate some contribution of fixed nitrogen (measured as transfer of $^{15}N_2$). This was combined with more common observations that inoculation, commonly and significantly, reduced the required doses of nitrogen fertilization for cultivation of many plant species. Evidence that nitrogen fixation contributes to the N balance of plants is based on the common observation of an increase in nitrogenase activity within inoculated roots, a microbial enzyme that does not exist in plants. This well-documented enzymatic activity in *Azospirillum* is of sufficient magnitude to account for the increase in total N yield of inoculated plants if *all* the fixed N is incorporated into the plants (Kennedy *et al.*, 1997; for earlier studies Bashan and Holguin, 1997 and references therein). On the other hand, many studies show that the contribution of nitrogen fixation by *Azospirillum* to the plant is minimal and ranged, at best, from 5% to 18% of the total N increase in the plant. In many

of those studies, the contribution was smaller than 5% or null. Hence, it is an open debate to this day (Bashan and Holguin, 1997; Bashan and Levanony, 1990; Bashan *et al.*, 2004). These findings almost caused an abandonment of nitrogen-fixation aspects of *Azospirillum*, except for continuing pure genetic studies.

Several confirmatory reports about the contribution of fixed nitrogen by *Azospirillum* to plants, similar in nature to reports of earlier years, illustrate the controversy. The ^{15}N isotope dilution technique indicated that there were significant biological N_2-fixation contributions to two genotypes of maize that showed similar increases in grain yield when they were inoculated with a mixture of *Azospirillum* strains or fertilized with the equivalent of 100 kg N ha^{-1}. These plant genotypes had a large increase in total N. This suggests that the yield response resulted from increased acquisition of nitrogen, but not from bacterial nitrate reductase (NR); NR^- mutants generally caused plant responses similar to those of the parent strains (Garcia de Salamone *et al.*, 1997). The ability of the bacteria to transfer fixed nitrogen from the atmosphere to wheat plants was tested using a $^{15}N_2$-enriched atmosphere. Labeled fixed nitrogen was detected in plant growth media and roots and shoots of wheat grown for 26 days in a $^{15}N_2$-enriched atmosphere, but the highest levels of ^{15}N were detected in wheat shoots. Ammonia or nitrate supplied to plants did not repress $^{15}N_2$-fixation (Ruppel and Merbach, 1997). Relationships of 12 *A. brasilense* strains with roots of a wheat cultivar were studied. They were compared for responses in root colonization, growth stimulation, and nitrogen supply to the plant. All strains colonized the root surface and interior. Most strains stimulated plant growth, but to different degrees. Some strains increased the total nitrogen in roots and leaves up to 80% over noninoculated plants, while others produced no effect on nitrogen content. Inoculation of five wheat cultivars with the most efficient strain for nitrogen fixation resulted in increased growth and nitrogen content, but the effects varied among the cultivars. These results suggest that a potential exists for *A. brasilense* to supply considerable nitrogen to wheat plants, probably dependent on specific bacteria–cultivar interaction (Saubidet and Barneix, 1998).

Apparently, dismissal of nitrogen fixation as a possible mechanism for promoting plant growth by *Azospirillum* in the 1990s was premature and additional greenhouse studies in the last decade showed significant and direct contribution of nitrogen fixation. Measurement of nitrogen fixation after inoculation with *A. lipoferum* and *A. brasilense* in rice showed that the N derived from the atmosphere were 20.0% (*A. lipoferum*) and 19.9% (*A. brasilense*) in basmati rice and 58.9% (*A. lipoferum*) and 47.1% (*A. brasilense*) in super-basmati rice (Mirza *et al.*, 2000). Using an *in vitro* model (*A. brasilense* and wheat) within 70 h after inoculation, insignificant amounts of newly fixed N were transferred from an ammonia-excreting strain of *A. brasilense* to the shoot tissue of wheat. Adding malate (a preferred

carbon source for *Azospirillum*), transfer of nitrogen to the shoots increased 48-fold, which indicates that 20% of nitrogen in the shoot was derived from nitrogen fixation. Apparently, the inability of the host plant to release sufficient carbon into the rhizosphere is a significant constraint on the development of the *A. brasilense*–wheat association. Perhaps wheat with an increased release of photosynthate to the rhizosphere should be a priority for improving effectiveness of the association (Wood *et al.*, 2001). Inoculation of strains of *A. amazonense* on rice increased grain dry matter and nitrogen accumulation at maturation. Contributions from nitrogen fixation were up to 27% of the contribution to the plant. Promotion of growth by *A. amazonense* for these rice plants was primarily a response to nitrogen fixation (Rodrigues *et al.*, 2008). Finally, winter wheat inoculated with *A. brasilense* having a point mutation in the ammonium binding site of glutamine synthetase showed the importance of its nitrogen contribution to the plant. The glutamine synthetase is one of the main ammonium-assimilating enzymes; mutations in this enzyme generally result in the release of ammonium from the bacterium to the environment. The ammonium-excreting mutant performed better than the wild-type *A. brasilense* strain for wheat growth parameters and yield (Van Dommelen *et al.*, 2009).

An innovative approach to enhance nitrogen fixation to plants by *Azospirillum* was the creation of a specialized site for nitrogen fixation, a para-nodule. This root structure externally resembles a legume nodule and can be induced by adding low concentrations of the auxin herbicide 2,4-D to roots (Tchan *et al.*, 1991). Because *Azospirillum* does not secrete significant amounts of ammonium and sometimes provides the plant only small amounts of nitrogen, spontaneous mutants of *A. brasilense* were selected that excrete substantial amounts of NH_4^+ and the bacteria were established inside para-nodules. When plants were grown on a nitrogen-free medium, these mutants were responsible for significant increases in organic matter (root and shoot dry matter and total plant nitrogen), compared with plants treated with wild-type *Azospirillum* or plants that were not inoculated. Analysis of $^{15}N_2$ in these plants showed that the mutants were able to transfer more nitrogen to the host plants than the wild-type strain (Christiansen-Weniger and van Veen, 1991). Para-nodules induced in rice seedlings were the preferential sites for colonization by a NH_4^+-excreting *A. brasilense* mutant. Nitrogenase activity in para-nodules structures inhabited by bacteria significantly increased, compared with untreated control plants (Christiansen-Weniger, 1997). It is probable that within para-nodules, bacterial nitrogenase is less sensitive to increased oxygen tension in the roots, as confirmed by Deaker and Kennedy (2001). Host plants benefit from enhanced nitrogen fixation in their roots with para-nodules because fixed nitrogen is incorporated into the host plant. Host plants probably stimulate nitrogenase activity of endophytic *Azospirillum* spp. by providing a carbon source as energy (Christiansen-Weniger, 1998).

These results show that the Gramineae are capable of establishing an association with diazotrophic bacteria in which ammonium-excreting bacteria provide the host plants with nitrogen. Para-nodule on wheat seedling roots was further developed by the researchers who invented it (Katupitiya *et al.*, 1995b; Sriskandarajah *et al.*, 1993) and specifically and consistently showed that nitrogenase activity in para-nodules was higher than in inoculated roots without para-nodules (Tchan *et al.*, 1991; Yu *et al.*, 1993; Zeman *et al.*, 1992). Similar results were obtained with maize (Saikia *et al.*, 2004, 2007). Para-nodules add a new dimension to research on biological nitrogen fixation, even if extensive developmental and biochemical modification of the para-nodule system is required before effective nitrogen fixation can be achieved. The options are intriguing (Christiansen-Weniger, 1994; Kennedy, 1994; Kennedy and Tchan, 1992).

In the last decade, perhaps as a response to the controversy mentioned earlier, studies have focused on the nitrogen cycle within cells of bacteria and on many details of molecular mechanisms and the genes involved that proliferate as *Azospirillum* was developed as a general model to study nitrogen fixation in nonsymbiotic bacteria. This is not a topic of this review (see e.g., Araujo *et al.*, 2004a,b; Huergo *et al.*, 2006a,b, 2009; Klassen *et al.*, 2005). The full genetic sequences of *A. brasilense* and *A. lipoferum* have been accomplished and they will be accessible at the Genoscope sites (France) and of *A. brasilense* at the Oak Ridge National Laboratory site (USA) (I. Kennedy, personal communication). Meanwhile, the complete nucleotide sequence of the *A. brasilense fixA*, *fixB*, *fixC*, and *fixX* genes were reported, as well as several other genes (Sperotto *et al.*, 2004). Mutants of the common *A. brasilense* strains Sp7 and Sp245 (defective in flocculation, differentiation into cyst-like forms, and colonizing of roots) had a higher nitrogenase expression than wild strains in association with wheat. Apparently, the ability of Sp7 and Sp245 mutants to remain in vegetative forms (spirillum and rods) improved their ability to express exceptionally high rates of nitrogenase activity. Restoring cyst formation and a normal colonizing pattern to the spontaneous mutant Sp7S reduced nitrogenase activity to the level of the wild Sp7. This suggests that bacterial cells in the vegetative state provides faster metabolism, which directly affects nitrogen fixation (Pereg-Gerk *et al.*, 2000). *A. brasilense* carrying *gfp* genes expressed pleiotropic physiological effects caused by disruption of the *clpX* gene encoding for heat-shock protein. One of the consequences of inserting the *gfp* gene is a threefold increase in nitrogen fixation (Rodriguez *et al.*, 2006). This phenomenon was confirmed in other *A. brasilense* strains (de Campos *et al.*, 2006). Apparently, higher expression of the *clpX* gene may be involved with creation of the Nif^- phenotype of the *A. brasilense* mutants by unknown mechanisms (Castellen *et al.*, 2009). Efficiency of nitrogen fixation and denitrification in *A. lipoferum* can be regulated by varying the concentration

of oxygen, nitrate, and molybdenum. The maximum growth rate in two strains was observed under microaerobic conditions, minimal nitrate, and the maximum concentration of molybdenum. These conditions were also conducive for obtaining maximum efficiency of denitrification (nitrate reduction to molecular N_2; Furina *et al.*, 1999). Microaerobic conditions favor nitrogen fixation. Low dissolved oxygen was also a limiting factor when ammonium concentrations limit growth of *A. lipoferum* (Tsagou *et al.*, 2003). In *A. brasilense*, cytochrome *c* oxidase is required under microaerobic conditions when a high respiration rate is needed. However, under nitrogen-fixing conditions, respiration rates do not seem to be a growth-limiting factor. Evidence for this was provided when a wild-type *A. brasilense* was compared with a *cytN* mutant *A. brasilense*. Under aerobic conditions, growth during the log phase was similar between the two types. Under microaerobic conditions (with NH_4^+ supplied; no nitrogen fixation), low respiration of *A. brasilense cytN* decreased its growth rate compared with the growth rate of the wild-type *A. brasilense*. Under nitrogen-fixing conditions (without NH_4^+ supplied), growth and respiration rates of the wild-type bacterium were significantly diminished and the differences in growth and respiration rates between the wild and mutant forms were smaller. Yet, the nitrogen-fixing capacity of the mutant was still approximately 80% of the wild-type (Marchal *et al.*, 1998). Out of 40 thermo-tolerant mutants developed from a mesophilic *A. lipoferum*, only 14 could grow and fix nitrogen at 45 °C. These mutants excrete ammonia only as very old cultures (maximum production after 12 days under stationary conditions; Steenhoudt *et al.*, 2001). Nitrogen fixation by aerobic bacteria is a very energy demanding process, requiring efficient oxidative phosphorylation, since O_2 is toxic to the nitrogenase complex. *Azospirillum* spp. and other well-known nitrogen-fixing soil bacteria have evolved a variety of strategies to deal with and overcome the apparent "O_2 paradox." The question is whether the specific environmental adaptations of azospirilla are sufficient to allow optimal proliferation and nitrogen fixation in their natural habitat. Could improving O_2-tolerance of the nitrogen-fixing process contribute to the development of more efficient strains for inoculation of plants (Marchal and Vanderleyden, 2000)? This remains a future research objective.

In evaluating the overwhelming data accumulated over the last 35 years on nitrogen fixation by *Azospirillum*, ignoring nitrogen fixation as a mechanism for *Azospirillum*, is premature. In several systems of inoculation, clear demonstration of significant increases of fixed nitrogen for plant growth was demonstrated, while it did not occur in others tests. It is also feasible that in systems where the contribution is small, the quantity of nitrogen provided by the nitrogen-fixing process is accumulative, with other mechanisms to produce the final growth promotion effect (see Section 3.6).

2.3. General improvement of root growth and enhanced uptake of minerals and water

Enhanced root systems, including root hairs, are the most common phenotypic phenomena observed following *Azospirillum* inoculation in most species. Consequently, improved root growth and function leading to improved water and mineral uptake was proposed in the late 1970s. Enhanced mineral uptake was a popular explanation for the inoculation effects in the 1980–1990s (for reviews, see Bashan and Holguin, 1997; Bashan and Levanony, 1990).

Increased mineral uptake in plants has been suggested due to a general increase in volume of the root system and not to any specific metabolic enhancement of the normal ion uptake mechanism (Morgenstern and Okon, 1987; Murty and Ladha, 1988) and that this is related to secretion of phytohormones by the bacteria. Other studies suggested a more active involvement in acquisition of minerals. Inoculation may promote availability of ions in the soil by helping the plant scavenge limiting nutrients (Lin *et al.*, 1983), which may explain the common accumulation of N compounds in the plant without any apparent N_2 fixation. Thus, the plant may absorb N more efficiently from the limited supply in the soil, resulting in a less N fertilization to attain a desired yield. By volume of information this is one of the largest parts of the *Azospirillum* literature, although the physiological, biochemical, and molecular details were left unsearched and only analyses of specific variables was presented. As a result, the available information about this mechanism is largely descriptive.

Several examples, out of many, illustrate the mechanism. In hydroponic systems in greenhouses, inoculation with *A. brasilense* increased the number and length of adventitious roots of *Sorghum bicolor* by 33–40% over non-inoculated controls, such as a higher rate of growth, earlier root appearance, and a greater elongation rate of individual roots (Sarig *et al.*, 1992). In addition to increasing (Kapulnik *et al.*, 1981, 1985b) or decreasing (Kucey, 1988) many root parameters, inoculation affected many foliage parameters. These changes were directly attributed to positive bacterial effects on mineral uptake by the plant. Enhancement in uptake of NO_3^-, NH_4^+, PO_4^{2-} K^+, Rb^+, and Fe^{2+} and several micronutrients by *Azospirillum* (Barton *et al.*, 1986; Jain and Patriquin, 1984; Kapulnik *et al.*, 1985a; Lin *et al.*, 1983; Morgenstern and Okon, 1987; Murty and Ladha, 1988; Sarig *et al.*, 1988) was proposed to cause an increase in foliar dry matter and accumulation of minerals in stems and leaves. During the reproductive period, these minerals could have been transferred to the panicles and spikes and result in higher yield and higher mineral content (Ogut and Er, 2006). Supporting evidence for increased mineral uptake by inoculated roots is provided by enhancement in proton efflux activity of wheat roots inoculated with *Azospirillum* (Bashan, 1990; Bashan *et al.*, 1989a). It is well known

that proton efflux activity is directly related to the balance of ions in plant roots (described below). Although some studies showed accumulation of N and minerals in the inoculated plants, others showed that enhanced growth of wheat and soybeans was not necessarily because of a general enhancement of mineral uptake (Bashan *et al.*, 1990).

In addition to improved mineral uptake, inoculation improved water status in stressed sorghum plants. Inoculated plants were less stressed, having more water in their foliage, higher leaf water potential, and lower canopy temperature than noninoculated plants. Soil moisture extraction by *Azospirillum*-inoculated plants was greater and water was extracted from deeper layers in the soil. Therefore, increased sorghum yield was primarily attributed to improved utilization of soil moisture (Sarig *et al.*, 1988; see Section 2.5 for more details.)

It is likely that improved mineral and water uptake occur in the *Azospirillum*–plant association. However, the descriptive data presented so far have not shown whether these improvements are the cause or the result of other mechanisms, such as changes in the balance of plant hormones or enhanced proton extrusion. Furthermore, the wide range of enzymatic activities related to these phenomena were poorly studied and no apparent evaluation of *Azospirillum* mutants deficient in induction of mineral and water uptake by plants has been made. Finally, it should be noted that very few strains have been studied and it is doubtful if all *Azospirillum* strains possess these abilities. There is evidence that some strains of *A. brasilense* failed to improve uptake of several ions, but nevertheless improved plant growth (Bashan *et al.*, 1990).

2.4. Phosphate solubilization and mobilization and rock weathering

Despite the reservations listed above, improved mineral uptake by plants was suggested as a major contribution of *Azospirillum* inoculation, therefore, azospirilla weathering of minerals in general and phosphorus in particular were studied. This has received attention because of the related larger field of phosphate solubilization that involves other bacterial genera.

A. halopraeferens, a bacterium that does not use glucose, and consequently does not produce acid, can solubilize insoluble inorganic phosphate *in vitro* by unknown mechanisms (Seshadri *et al.*, 2000). Two strains of *A. brasilense* and one strain of *A. lipoferum* were capable of producing gluconic acid, thereby leading to solubilization of insoluble phosphate in rocks (Puente *et al.*, 2004a; Rodriguez *et al.*, 2004). Sugars, like glucose, are part of the root exudates of pea plants grown in P-deficient substrates and enhanced the capacity of *Azospirillum* spp. to solubilize normally insoluble $Ca_3(PO_4)_2$. The relative proportion of glucose in pea exudates decreased under P deficiency, while the content of galactose, ribose, xylose, and fucose increased. *Azospirillum* spp. can metabolize all these sugars. Therefore, the shift in sugars under

P deficiency increased the capability of *Azospirillum* spp. to mobilize phosphate (Deubel *et al.*, 2000). Similarly, inoculation of cardon (a giant cactus) with *A. brasilense* Cd enhanced phosphate solubilization and enhanced plant growth (Carrillo *et al.*, 2002).

These observations can partly be explained by acidification of the nutrient medium by protons and organic acids. *Azospirillum* spp. can produce different organic acids that assist in P solubilization, depending on the sugar in the root exudates. Yet, *Azospirillum* can solubilize P by itself without adding root exudates. For example, three *Azospirillum* strains were isolated from the ectomycorrhizal sporocarps (*Rhizopogon vinicolor*) that colonized Douglas fir trees. *In vitro*, they were able to degrade limestone, marble, and calcium phosphate (Chang and Li, 1998). These observations were confirmed using other strains of *Azospirillum* (Puente *et al.*, 2004a,b, 2006). Uptake by *A. brasilense* cells of the essential elements Mg, Ca, Mn, and Fe and trace elements V, Co, Ni, Cu, Zn, and Pb (which do not essentially suppress growth of bacterial cultures) present in weathered rock fragments and are accumulated by the cells was shown. Zn and Cu were accumulated in the bacterial biomass in relatively significant amounts, but uptake of Co and Ni was much less, and Pb and V were apparently not assimilated by azospirilla. In particular, Cu cations were effectively absorbed by the bacterium and this increased the rate of uptake of other metals; however, the process takes time. Short exposures have only a limited effect on absorption of Cu (Ignatov *et al.*, 2001; Kamnev *et al.*, 1997a). Additionally, these bacteria are capable of producing structural modifications of the magnesium-ammonium orthophosphate molecule when added to the medium (Kamnev *et al.*, 1999a). Fourier transform infrared spectroscopy is a powerful tool for nondestructive identification and characterization of cell components; it was applied to studies of molecular structures in *A. brasilense*, its essential element content (Kamnev *et al.*, 1997b, 2001), heavy metal-induced metabolic changes in the cells (Kamnev *et al.*, 2002), and membrane composition and structure (Kamnev *et al.*, 1999b). These capabilities notwithstanding, it has not been demonstrated so far that these elements, obtained from the environment, were transferred to the plant.

The research field of mineral solubilization and mobilization in *Azospirillum* is potentially useful for studying interactions and survival of the bacteria in the soil. Although the literature treated this proposal as an individual entity, it should be considered as a subfield of enhanced mineral uptake mentioned earlier.

2.5. Mitigation of stresses

From the earliest field experiments with *Azospirillum* in the 1980s, the best effects on plant growth and yield were obtained when the growth conditions were suboptimal. A common explanation for the effects of *Azospirillum* on

plant growth was reduction in environmental stresses by the bacteria, providing the plant a more favorable environment to grow in an otherwise limiting environment. Sometimes inoculation permits plant growth in soils that normally did not allow growth. None of these theories can explain enhanced growth of inoculated plants under favorable plant growth conditions that also occurred and were regularly reported. Environmental stressors varied and included mitigation of drought (Sarig *et al.*, 1990), salinity (Creus *et al.*, 1997), heavy metals (Belimov and Dietz, 2000), toxicity of other substances (de-Bashan and Bashan, 2008), extreme pH (de-Bashan *et al.*, 2005), toxic humic substances (Bacilio *et al.*, 2003), and suboptimal levels of nitrogen (discussed earlier).

2.5.1. Salinity stress

Numerous cultivated soils worldwide are becoming more saline, mainly from the use of marginal irrigation water, from excess fertilization, and various desertification processes. Inoculation with *Azospirillum* sp. under saline stress conditions is therefore commonplace. Prior findings (for a review, see Bashan and Holguin, 1997) showed that common agricultural *Azospirillum* strains tolerated high salinity (~2%). Salt resistance among species increased from *A. amazonense* (lowest) to *A. halopraeferans* (highest), the latter tolerating over 3% NaCl (seawater salinity).

Azospirillum inoculation of maize at NaCl concentrations up to –1.2 MPa significantly increased chlorophyll, K, Ca, soluble saccharides, and protein contents, compared with control maize growing without NaCl (Hamdia and El-Komy, 1997). Alleviation of salt stress in maize involved several changes that probably were related to different operating mechanisms: proline concentration declined significantly, the concentration of most amino acids increased on exposure to NaCl, as well as when inoculated with *Azospirillum*. *Azospirillum* apparently restricted Na^+ uptake and enhanced the uptake of K^+ and Ca^{2+}. Finally, inoculation stimulated nitrate reductase and nitrogenase activity in shoots and roots (Hamdia *et al.*, 2004). Inoculating wheat seedlings with *A. brasilense* exposed to severe salt (NaCl) or osmotic (polyethylene glycol) stress significantly reversed part of the negative effects; both stresses reduced the relative elongation rate of shoots. Fresh weight, fresh weight/dry weight ratio, water content, and relative water content were higher in shoots from inoculated plants than in stressed controls (Creus *et al.*, 1997). Similarly, under high NaCl concentration, inoculation of wheat with *A. lipoferum* reduced some of the deleterious effects of NaCl (Bacilio *et al.*, 2004). Finally, *Azospirillum*-inoculated lettuce seeds had better germination and vegetative growth than noninoculated controls after being exposed to NaCl (Barassi *et al.*, 2006).

The most fundamental omissions in current knowledge are (1) uncertainty about whether improved salt tolerance of the bacterium is needed to enhance the bacterium's effect on plants or if existing salt tolerance in plants

is adequate to ensure positive growth-promotion by inoculation; (2) what are the mechanisms that are triggered and are responsible for enhanced saline resistance after inoculation; and (3) what is the microbial mechanism that provides resistance in plants.

2.5.2. Water stress

Apparently, inoculation with *Azospirillum* improved growth under water-stress conditions as was initially demonstrated in the 1980s (for a review, see Bashan and Levanony, 1990). Subjecting inoculated *S. bicolor* plants to osmotic stress in hydroponic systems diminished the adverse effects caused by osmotic stress, such as reduction of leaf senescence (Sarig *et al.*, 1990). Coleoptile height and fresh and dry weight of wheat seedlings inoculated with *A. brasilense* Sp245 were enhanced, despite the water stress (Alvarez *et al.*, 1996). Inoculation with *Azospirillum* alleviated the stress on wheat plants grown under drought conditions (El-Komy *et al.*, 2003). Turgor pressure at low water potential was higher in inoculated seedlings in two wheat cultivars under osmotic stress. This could result from better water uptake as a response to inoculation that, in turn, is reflected by faster shoot growth in inoculated seedlings exposed to these stresses. They showed better water status and effects on cell wall elasticity or apoplastic water (Creus *et al.*, 1998). To assess the contribution of *A. brasilense* Sp245 during drought when flowers open (anthesis), inoculated wheat seeds were subjected to drought. Even though all the plants underwent osmotic stress, significantly higher water content, relative water content, water potential, apoplastic water fraction, and lower cell wall modulus of elasticity values were obtained in inoculated plants. Grain yield loss to drought in inoculated plants was significantly reduced and significantly higher Mg, K, and Ca in grains were detected. Probably, inoculation improved water status and an additional "elastic adjustment" in plants (Creus *et al.*, 2004). Recently, inoculation with *A. brasilense* contributed to protection of wheat seedlings under water stress through changes in the fatty acid distribution profiles of phosphatidylcholine and phosphatidylethanolamine, major root phospholipids (Pereyra *et al.*, 2006). Transformed *A. brasilense* that could produce trehalose, an osmotic-regulating sugar, was more salt resistant than the wild type and significantly enhanced the survival of maize growing under drought stress. It also significantly increased biomass and leaf and root length of the plants (Rodríguez-Salazar *et al.*, 2009). The limitations in our knowledge regarding the effect of inoculation under saline stress are valid for osmotic stress, as well.

2.5.3. Herbicides

Cotton plants could be partly protected from harmful effects of the herbicide 2,4-D by inoculation with *A. brasilense*. The degrading plasmid of 2,4-D was transferred into *A. brasilense* Sp7. Trans-conjugants degraded

2,4-D in pure culture via cometabolism. However, when the transconjugants were inoculated on cotton seeds, the plants were resistant only to low levels of the herbicide, which is not sufficient for protection of cotton. Plants growing in soils with this concentration of herbicide and inoculated with wild-type strains died (Feng and Kennedy, 1997).

2.5.4. Toxic metals

Another possible mechanism for producing a healthier plant is reduction of metal toxicity in contaminated soils and mine tailings that, under normal conditions, almost completely inhibits plant growth. Although the bacterium tolerate only moderate levels of metals and other toxic compounds (see previous reviews Bashan and Holguin, 1997; Bashan and Levanony, 1990; Bashan *et al.*, 2004; also Kamnev *et al.*, 2005, 2007), it apparently contributed mechanisms allowing plants to grow in mine tailings or contaminated soils. Cadmium causes severe inhibition of growth and nutrient uptake in barley. In the presence of $CdCl_2$, inoculation with *A. lipoferum* partly decreased Cd toxicity, possibly through the improvement of mineral uptake. Additionally, inoculation slightly enhanced root length and biomass of barley seedling treated with Cd and the amount of nutrients absorbed by the inoculated plants increased significantly. There was only some protection against Cd toxicity, but no uptake of Cd, since Cd content in the inoculated plants was unchanged (Belimov and Dietz, 2000; Belimov *et al.*, 2004). *A. brasilense* Sp245 associated with wheat changes the speciation, bioavailability, and plant uptake of arsenic. Plants inoculated with *Azospirillum* accumulated less arsenic than did uninoculated plants (Lyubun *et al.*, 2006). Inoculation of the wild desert shrub quailbush (*Atriplex lentiformis*) growing in extremely stressed environment with *A. brasilense* strains Sp6 and Cd, such as acidic mine tailings having high metal content, resulted in a significant increase in production of plant biomass (L.E. de-Bashan *et al.*, unpublished data). Similar results were obtained when wild yellow palo verde desert trees (*Parkinsonia microphylla*) were inoculated with *A. brasilense* Cd in rock phosphate tailings (Bashan *et al.*, unpublished data).

2.5.5. Compost and humic substances

Some compost may be toxic to plants because of elevated humic acids or inappropriate preparation. Inoculation of wheat seeds with *A. brasilense* or *A. lipoferum* prior to sowing in soil that was amended with two types of compost improved seed germination and plant development. The bacteria possibly changed or consumed the humic acids because both bacterial species can survive and grow in high humic acid solution as the sole source of carbon; thus, modify the composition of the compost during *in vitro* tests (Bacilio *et al.*, 2003).

2.5.6. pH and toxic substances in aquatic environments

Apart from terrestrial applications, *Azospirillum* is being used as an inoculant in aquatic environments mainly to promote the growth and metabolism of microalgae of the genus *Chlorella* that is used in wastewater treatment (de-Bashan *et al.*, 2004; Gonzalez and Bashan, 2000; Hernandez *et al.*, 2006). Under aquatic conditions, the pH, available dissolved nutrients, and toxic molecules to the microalgae have significant impact on the process of mass production. High pH of the medium interferes with the microalgal cell cycle and decreases microalgal population. Coculturing of the microalgae with *A. brasilense* eliminated this negative effect (de-Bashan *et al.*, 2005). Similarly, high levels of the amino acid tryptophan reduced multiplication of *C. vulgaris* where coculturing with *A. brasilense* significantly reduced the inhibition probably by converting it to IAA that enhances the growth of the microalgae (de-Bashan and Bashan, 2008).

2.5.7. Protection from relative high light intensities

Inoculation of plants sometimes occurs under light intensity that is stressful and has an inhibiting effect on specific crops. Inoculation of wheat seedlings with *A. brasilense* Cd significantly increased the quantity of the photosynthetic pigments chlorophyll *a* and *b*, but also the auxiliary photoprotective pigments violaxanthin, zeaxanthin, antheroxanthin, lutein, neoxanthin, and β-carotene that help the plant to sustain photosynthesis under unfavorable light conditions. This outcome yielded greener plants with no apparent visible stress. The greatest difference in the quantity of all pigments between inoculated and noninoculated plants occurred in the first week of growth (Bashan *et al.*, 2006). Similarly, although the microalgae *C. sorokiniana* is capable of growing at high light intensities, coculturing with *A. brasilense* enhanced this capacity and the microalgae could tolerate extreme light intensities as high as 2500 μmol m^{-2} s^{-1} (de-Bashan *et al.*, 2008b).

Taking all these phenomena together, it appears that a multitude and remotely related or unrelated mechanisms are operating in these complex interactions of *Azospirillum* with plants. All these accumulating findings yielded a recent proposal to include *Azospirillum* in the group of other rhizosphere PGPB that regulated homeostasis of plants under conditions of abiotic stress. This group was designated "Plant Stress-Homeo-regulating Bacteria" (PSHB; Cassan *et al.*, 2009b; Sgroy *et al.*, 2009). These types of bacteria, *Azospirillum* included, may use an assortment of mechanisms, such as biosynthesis of phytohormones, growth regulators, osmoregulator molecules, expression of specific regulatory and metabolic enzymes, and immobilization or catabolism of various toxic molecules for plants to assist plant growth. This proposal forms a part of the initial theory of Section 3.6.

3. Other Proposed Mechanisms

3.1. Biological control

Azospirillum is not yet known as a typical biocontrol agent of soil-borne plant pathogens because many strains lack direct suppressive chemicals or hydrolytic enzymes likely to affect plant pathogens. However, reports are accumulating that this mechanism has been overlooked. Some possible mechanisms used by *Azospirillum* to reduce damage from pathogens have been demonstrated as environmental competition and displacement of pathogens, inhibition of seed germination of parasitic weeds, general enhancement of plants to resist pathogen infection, and possible inhibition of fungal growth via production (at least *in vitro*) of microbial toxic substances.

3.1.1. Toxic substances

When iron was withheld, *A. lipoferum* strain M produced catechol-type siderophores under iron starvation that exhibited antimicrobial activity against various bacterial and fungal isolates (Shah *et al.*, 1992). Although some strains from Brazil produce cyanide (HCN) *in vitro* (Gonçalves and de Oliveira, 1998), this feature is uncommon in strains from other geographic locations. Some *Azospirillum* isolates produced bacteriocins that inhibited growth of several indicator bacteria (Tapia-Hernandez *et al.*, 1990). An antimicrobial auxin-like molecule, phenylacetic acid was isolated from an *A. brasilense* culture (Somers *et al.*, 2005). *A. brasilense* cells contain a low molecular-weight compound that inhibits germination and growth of the radicle of Egyptian broomrape seeds (*Orobanche aegyptiaca*), a specific weed parasite of sunflower (Dadon *et al.*, 2004). *Azospirillum* spp. inhibited germination of the parasitic striga weed (witchweed) seeds (*Striga hermonthica*) that infest fields of tropical sorghum, thereby promoting growth of sorghum (Bouillant *et al.*, 1997). *Azospirillum* cells suspended in a synthetic germination stimulant did not inhibit germination of striga weed seeds, but blocked radicle elongation. These radicles had abnormal morphology and contained no vacuolated cells in the root elongation zone. Lipophilic compounds extracted from the medium of bacteria prevented germination of striga seeds (Miché *et al.*, 2000). So far, *Azospirillum* has not been reported to induce any negative effect on healthy plants (Bashan, 1998). If this is the case, these toxic compounds are either *in vitro* artifacts or are induced only in the presence of pathogens.

3.1.2. Competition

The effect of *A. brasilense* on crown gall formation in Dicotyledoneae was studied after inoculation with virulent strains of *Agrobacterium tumefaciens*. When wounded tissues of grapevines and carrot disks were inoculated with live cells of *A. brasilense* strains 94-3 or Sp7, development of the typical

bacterial galls was inhibited and the protective effect of *Azospirillum* lasted over a 24-h period (Bakanchikova *et al.*, 1993). When *A. brasilense* Cd was added to a culture with the pathogenic mangrove rhizosphere bacterium *Staphyloccus* spp., the population of the latter was significantly reduced (Holguin and Bashan, 1996).

To assess displacement of pathogens by inoculation with *Azospirillum*, the tomato leaf pathogen *Pseudomonas syringae* pv. *tomato* (PST, bacterial speck) and *A. brasilense* were inoculated onto tomato plants, as a mixed culture or consecutively. Inoculation of seeds with a mixed culture resulted in reduction of the pathogenic population in the rhizosphere, increased the population of *A. brasilense*, prevented development of PST, and improved plant growth. PST did not survive in the rhizosphere in the presence of *A. brasilense*. Inoculation of leaves with the mixed bacterial culture under mist conditions significantly reduced the population of PST and significantly decreased the severity of the disease. Challenge with PST after *A. brasilense* was established in the leaves further reduced PST and severity of the disease and significantly enhanced plant development. Selective enhancement of the population of *A. brasilense* on leaves occurred by applying malic acid (favorable for *A. brasilense*, but not for PST), decreased PST to almost undetectable levels, almost eliminated disease development, and improved plant growth to the level of uninoculated healthy controls (Bashan and de-Bashan, 2002a). Seeds inoculated with *A. brasilense* Sp7 and later challenged by two foliar bacterial pathogens of tomato (*Clavibacter michiganensis* spp. *michiganensis* [bacterial canker] and *Xanthomonas campestris* pv. *vesicatoria* [XCV, bacterial spot]) delayed leaf- and plant-death compared with untreated controls, but canker severity was not affected. Unfortunately, inoculation with *Azospirillum* increased the severity of XCV on cherry tomatos (Romero *et al.*, 2003). Several isolated bacterial strains showed antagonism toward the fungus *Aspergillus flavus* that produces aflatoxin (the most potent carcinogenic mycotoxin produced by some fungi), and were capable of degrading the toxin *in vitro*. Since identification of the microorganism was based on morphological characteristics, it is uncertain whether the identification of the strains as *Azospirillum* is valid (Cho *et al.*, 2000). A strain of *A. brasilense* with increased capacity for N_2-fixation was tested *in vitro* against the soil-borne plant pathogens, *Fusarium oxysporum* f. sp. *lycopersici*, *Rhizoctonia solani*, and *Pythium* sp. that infect cucumbers. The bacteria reduced the dry weight of *Fusarium* mycelium by 90–96%, of *Rhizoctonia* by 72–94%, of *Pythium* by 71–95%, and completely eliminated *Sclerotinia* mycelium (Hassouna *et al.*, 1998).

3.1.3. Production of a "healthier plant" by unknown mechanisms

Many examples of possible "biological control" are reported without specifying the mechanisms. It is assumed that inoculation produce healthier plants by deterring pathogenic infections (Tilak *et al.*, 2005). This is a

possibility especially when the effect recorded is against microfauna and insects and not against microorganisms, as is expected.

For example, inoculation with *A. lipoferum* of mung bean (*Vigna radiata*) infected with root-knot nematode (*Meloidogyne incognita*) led to fewer root galls and egg masses per root system. After inoculation with *A. lipoferum*, plants infected with the nematode had significantly greater growth and biomass, probably related to a greater number of functional nodules on roots that had been infected (Khan and Kounsar, 2000). When inoculated with *A. brasilense*, okra (*Abelmoschus esculentus*) showed enhanced plant characteristics and pod yield. At the same time, there was a significant reduction in root-knot nematodes egg masses, eggs per egg mass, and total nematode population (Ramakrishnan *et al.*, 1997). Similar results in sunflower were obtained with a commercial inoculant of *A. brasilense* (Ismail and Hasabo, 2000). Maize that was inoculated with a combination of mycorrhizal fungi, *Glomus fasciculatum*, *Azospirillum* sp., and phosphate-solubilizing bacteria reduced the population of the *Pratylenchus zeae* nematode and induced very high cob yield (Babu *et al.*, 1998). When *A. lipoferum* was inoculated onto wheat plants, it reduced *Heterodera avenae* nematode infection (Bansal *et al.*, 1999). Inoculation of sorghum with *A. brasilense* to control the sorghum shoot fly *Atherigona soccata* that causes dead-heart in sorghum resulted in a 10-fold reduction of the disease and increased grain yield (Kishore, 1998).

A. brasilense was applied as a foliar spray against foliar fungal and bacterial diseases of mulberry, such as powdery mildew caused by *Phyllactinia corylea*, black leaf spot caused by *Pseudocercospora mori*, black leaf rust caused by *Cerotelium fici*, and bacterial leaf blight caused by *P. mori*. Inoculation reduced fungal pathogens and excelled as a treatment against bacterial blight (Sudhakar *et al.*, 2000). The addition of *Rhizobium*, *Azospirillum*, or *Azotobacter* inocula as a combined seed and soil treatment in cultivation of pearl millet (*Pennisetum glaucum*) reduced downy mildew (*Sclerospora graminicola*) in the leaves (Gupta and Singh, 1999). Inoculation with arbuscular mycorrhizal (AM) fungi and *Azospirillum* spp. suppressed damping-off disease in chili (*Capsicum* sp.) caused by *Pythium aphanidermatum* (Kavitha *et al.*, 2003). Combinations of several ineffective management tactics (spraying Cu and streptomycin combined with *Azospirillum* seed inoculation and seed disinfections, individually ineffective against PST, significantly reduced occurrence and severity caused by PST and also improved plant growth. Additionally, the combined treatment significantly reduced the amount of chemical pesticides required to protect tomato plants from PST (Bashan and de-Bashan, 2002b). The mechanisms by which this happens in all the described cases remain unknown.

So far, *Azospirillum* is not commonly reported to induce systemic resistance in plants. However, inoculation of rice plant with the endophyte *Azospirillum* sp. B510 induced disease resistance against diseases caused by

the virulent rice blast fungus *Magnaporthe oryzae* and bacterial pathogen *Xanthomonas oryzae*, apparently by activating a novel type of resistance mechanism independent of salicylic acid-mediated defense that does not signal accumulation or expression of pathogenesis-related genes (Yasuda *et al.*, 2009).

At this time, these reports do not provide conclusive evidence that *Azospirillum* is a true biological control agent, although significant biological control activity can be attributed to this genus.

3.2. Nitric oxide

Nitric oxide (NO) is a volatile, lipophilic free radical which participates in metabolic, signaling, defense, and developmental pathways in plants (Cohen *et al.*, 2010; Lamattina and Polacco, 2007; Lamattina *et al.*, 2003). As its major role, NO participates in the IAA signaling pathways. This participation leads to lateral and adventitious root formation where the exact role of NO is as an intermediary in IAA-induced root development (Correa-Aragunde *et al.*, 2004, 2006; Pagnussat *et al.*, 2002, 2003).

One wild-type *A. brasilense* Sp245 can produce NO *in vitro*, under anoxic and oxic (or aerobic) conditions (Creus *et al.*, 2005). The latter can be achieved by possible different pathways, such as aerobic denitrification and heterotrophic nitrification. NO is produced during the middle and late logarithmic phases of growth (Molina-Favero *et al.*, 2007, 2008). An NO-dependent promoting activity in *A. brasilense* Sp245 induces morphological changes in tomato roots regardless of the full bacterial capacity for IAA synthesis. An IAA-attenuated mutant of this strain, producing up to 10% of the IAA level compared with the wild-type strain (Dobbelaere *et al.*, 1999) had the same physiological characteristics and slightly less effect on root development. When the NO was removed, using a chemical NO scavenger, both types of root formation were inhibited. This demonstrates that NO-mediated *Azospirillum* induced branching of roots. These results provide further evidence of an NO-dependent promoting activity of tomato root branching, regardless of the bacterium's capacity for synthesizing IAA (Molina-Favero *et al.*, 2008), a phenomenon that occurs in other inoculation systems lacking IAA activity (see above). It is commonly argued that denitrification in agriculture is considered, in general, and specifically in plant inoculations, as an undesirable feature of PGPB because it reduces availability of N (Zimmer *et al.*, 1984) for the plant. Yet, the capacity of *A. brasilense* to reduce nitrate aerobically to NO, which in turn, could promote growth of tomato roots is a point for reconsideration.

Several studies demonstrate the continuous relation between NO and IAA on root development (Huang *et al.*, 2007; Lombardo *et al.*, 2006; Tewari *et al.*, 2007). It is possible that a connection, not proven so far, exists in *Azospirillum*–plant systems. However, the way that IAA and NO

are acting together, if acting, on plant cells triggering the branching of roots is still an open question for research. Nonetheless, the relationship between NO and *A. brasilense* showed that, in addition to the well-established connection between NO production and defense responses to pathogenic microorganisms (Modolo *et al.*, 2005; Zeidler *et al.*, 2004), it seems that NO metabolism also plays a role in the positive close association of PGPB with roots.

3.3. Nitrite

Nitrite (NO_2^-), either directly added or excreted by *A. brasilense* during nitrate respiration may participate in growth promotion effects. It causes a sharp increase in the formation of lateral roots (Zimmer *et al.*, 1988). Nitrite is produced under anaerobic or microaerobic conditions by the dissimilatory nitrate reduction pathway, in addition to NO and nitrous oxide (N_2O; Hartmann and Zimmer, 1994). Nitrite could have promoting effects when reacting with ascorbate (Bothe *et al.*, 1992; Zimmer *et al.*, 1988). This avenue has not been investigated further.

3.4. Signal molecules and enhanced proton extrusion from roots

Whatever the operating mechanism, *Azospirillum* affects plant cell metabolism from outside the cell (without entering the intact plant cells) and this suggests that these bacteria are capable of excreting and transmitting a signal (s) that crosses the plant cell wall and is recognized by the plant membranes. This interaction can initiate a chain of events resulting in altered metabolism of the inoculated plant and proliferation of roots. Since plant membranes are extremely sensitive to any change, their response may serve as a precise indicator of *Azospirillum* activity at the cellular level. Improving plant growth by affecting proton and organic acid extrusion (proton pump) mechanisms in plants by inoculation with *Azospirillum* spp. was proposed two decades ago.

A proton pump is an integral membrane protein that is capable of moving protons (H^+) across the membrane of a cell, mitochondrion, or other subcellular compartment. In cell respiration, the pumps move protons from the space enclosed by the two membranes within the organelle and release the protons into the intermembrane space. The confined protons create a gradient in both pH and electrical charge across the plasma membrane that acts as a reservoir of stored energy for the cell. For plants to react to their constantly changing environments and simultaneously maintain optimal metabolic conditions, the expression, activity, and interplay of the pumps generating these H^+ gradients have to be tightly regulated (Gaxiola *et al.*, 2007; Schumacher, 2006). Additional functions, such as opening and

closing of stomata, cell growth, and intracellular pH homeostasis, have been proposed (Duby and Boutry, 2009). Short exposure of wheat roots to live *A. brasilense* Cd significantly enhanced the proton efflux of the root at 5 h after inoculation. Bacteria in the logarithmic phase are required for this enhancement, which is a triggering nature (Bashan, 1990; Bashan *et al.*, 1989a). Inoculation of soybean seedlings with the same *Azospirillum* strain significantly reduced the membrane potential in every root part and this was greatest in the root elongation zone (Bashan, 1991; Bashan and Levanony, 1991). Inoculation of soybeans and cowpeas with this strain increased proton efflux from their roots and changed the phospholipid content in membranes of cowpeas (Bashan *et al.*, 1992). Although the nature of the released signal molecule is still unknown, *Azospirillum* probably targets plant membranes on plant roots. This phenomenon also occurs in cardon cactus. Lowering the pH of the rhizosphere increases the availability of phosphorus and iron to plants, especially in arid lands with high calcium content and soil pH (Carrillo *et al.*, 2002). A confirmatory study of the proton extrusion phenomenon in wheat showed that inoculation enhanced proton efflux and elongation of the roots. Although the evidence is circumstantial, perhaps these two phenomena are related. This effect was directly dependent on the bacterial strain–plant combination, suggesting that compatible strains are necessary to induce this activity (Amooaghaie *et al.*, 2002). This kind of investigation has not been pursued in recent years.

It is possible that a receptor in *A. brasilense* is involved in the binding of wheat germ agglutinin (WGA; one of the most studied plant lectins; Antonyuk *et al.*, 1993). This binding induced changes in the cellular metabolism of *A. brasilense* Sp245 and promoted nitrogen fixation, excretion of ammonium ions, and synthesis of IAA (Antonyuk and Evseeva, 2006; Antonyuk *et al.*, 1993, 1995). WGA changed the relative proportion of acidic phospholipids of the membrane. It is possible that acidic phospholipids participate in trans-membrane communication. WGA may function as a signal molecule in the *Azospirillum*–plant association (Antonyuk *et al.*, 1995). Some *Azospirillum* strains are known to produce several lectins *in vitro* (Castellanos *et al.*, 1998). Two cell-surface lectins isolated from *A. brasilense* Sp7 and from a mutant (defective in hem-agglutinating activity), *A. brasilense* Sp7.2.3, affected activities of α-glucosidase, β-glucosidase, and β-galactosidase in the membrane and apoplast fractions of roots of wheat seedlings (Alen'kina *et al.*, 2006). Other lectins induced changes in the mitotic state of growing onion plant cells (Nikitina *et al.*, 2004).

In general, effects on proton extrusion merit further investigation because the changes in the metabolism of the roots may induce enhanced mineral and water uptake even without proliferation of roots that are induced by phytohormones. This may provide further support to the theory of enhanced mineral uptake in cases when hormonal activity is not detected.

3.5. *Azospirillum* nitrate reductase

An alternative to nitrogen fixation as an explanation for N accumulation after inoculation of wheat plants by *Azospirillum* is the bacterial NR theory. NR activity in wheat leaves was decreased by inoculation with some *Azospirillum* strains. Inoculation with NR^- mutants resulted in a small response, concomitant with an increase in leaf NR, compared with inoculation with the parental NR^+ strain (Ferreira *et al.*, 1987). Inoculation of field-grown plants with *A. brasilense* Sp245 and its NR^- mutant confirmed that the mutant was significantly less effective in increasing yield than the parental strain (Boddey and Döbereiner, 1988). This phenomenon indicates that the effect of some *Azospirillum* strains on wheat plants is not solely via nitrogen fixation (both the parental and the mutant strains have this ability), but rather results from an increase in assimilating nitrate. The parental strain aided reduction of nitrate in the roots and thus decreased translocation of nitrate to the leaves, while inoculation with the NR^- mutant caused direct translocation and reduction of nitrate in the foliage. This theory might explain, in part, the observation of increased N accumulation in shoots because the unaffected ability to fix nitrogen may also contribute N to the plants in addition to NR activity. It also might be a part of a larger theory of enhanced mineral uptake by *Azospirillum* inoculation (described earlier). This line of research has not been pursued further.

3.6. Additive hypothesis

Several recent studies on modes of action in *Azospirillum* gave new momentum to the additive hypothesis that was suggested 20 years ago. The hypothesis considers multiple mechanisms rather than one mechanism participating in the association of *Azospirillum* with plants. These mechanisms operate simultaneously or in succession, the contribution of an individual mechanism being less significant when evaluated separately. The sum of activities under appropriate environmental conditions results in the observed changes in plant growth (Bashan and Levanony, 1990). For example are the cases where nitrogen fixation contributes less than 5% of the observed effect of *Azospirillum* on the plant. As such low levels, it is not sufficient and does not fully explain increases in yield. When combined with other small mechanisms, this may be a significant contribution. With a general mechanism unknown, or more likely, does not exist after 30 years of intensive research, it would be more practical to look at the effects of *Azospirillum* spp. on the whole plant as an outcome of multiple mechanisms rather than a single mechanism operating at the organ, tissue, cellular, or molecular levels. Support for this notion is provided by an analysis of literature of many of the known cases of the effect of inoculation on the root to shoot (S/R) ratio that shows that the general effect of *Azospirillum*

spp. on the entire plant was largely overlooked. From the changes the bacteria produce in the S/R ratio, it appears that it also participates in the partitioning of carbon compounds within the plant, a phenomenon that is well recognized as multiparametric. The analysis provides supportive experimental data (although collected from many diverse studies) that indicate that the mode of action of *Azospirillum* spp. is probably composed of multiple mechanisms (Bashan and Dubrovsky, 1996). Additional supportive experimental data are provided by recent studies on polyamines (Cassan *et al.*, 2009a) and nitrogen fixation (Van Dommelen *et al.*, 2009) that were presented earlier.

4. Concluding Remarks and a Proposal

Today, the prevailing explanation for the effect of *Azospirillum* on plants is the production of an assortment of phytohormones, mainly IAA, altering the metabolism and morphology of the roots, yielding better mineral and water absorption, hence, higher yields. The contribution of nitrogen fixation is more controversial and, despite the increasing large volume of literature on other possible mechanisms, these are largely ignored by reviews on the topic of plant growth promotion, mostly evaluating PGPB in general.

In a comprehensive analysis of the knowledge about physiology, metabolic pathways, and molecular biology mechanisms of *Azospirillum* and their possible mode of action, it is apparent that phytohormones, especially IAA working in synchronization with other phytohormones produced by the bacterium, play a major role in various aspects of metabolism for growth. However, to attribute extremely complex phenomena for nonspecific causes of growth promotion in numerous plant species inoculated with many strains of *Azospirillum* having great differences in physiological traits, to one or a few substance (s) produced in abundance, mainly *in vitro*, is an oversimplification. Yet, it is, useful research tool for probing the mode of action of these bacteria.

There was, and still is, a disproportion between the large amount of knowledge on the bacterium cell and less knowledge about its interaction with the plants. In many aspects of interaction, such as mitigation of stresses or biological control, our knowledge about the mode of action is close to nil. Unfortunately but frequently, the knowledge about bacterial metabolisms *per se* is extrapolated to explain possible effects on plants without providing solid evidence that such activity do exist *in planta*.

Mutants that are defective in several traits are used in this field of research, but are employed on a smaller scale than in the related fields such as biological control of plant pathogens. For a more accurate determination of the role of phytohormones in promoting growth in general, and

IAA in particular, there is a need to obtain a mutant that is totally deficient in IAA production, but otherwise identical to the parent strain. Although several IAA-attenuated mutants were constructed, this goal has not yet been achieved. The same is true for other phytohormones. Additionally, to clearly state whether hormones are the main mechanism for promoting growth, we need to demonstrate that other proposed mechanisms have a minor role. Yet, there is much evidence to the contrary. These include the importance of nitrogen fixation under specific circumstances, including the postpara-nodule colonization (presented earlier) and new data collected under greenhouse and field conditions (Rodrigues *et al.*, 2008; Van Dommelen *et al.*, 2009). The overall accumulated evidence that nitrogen fixation plays a role in the association reconfirms that dismissal of nitrogen fixation, as a mechanism for plant growth reported in several reviews in recent years, is premature, and that nitrogen fixation should be reconsidered as a plausible comechanism. Additionally, the importance of signal molecules in initiating the cascade of events that induce a plant response, should be considered, perhaps in relation to root membranes (the main subcellular units responsible for mineral uptake detected in numerous associations of plant with *Azospirillum*). Many cases of mitigation of environmental stresses, possibly by mechanisms not envisioned so far or by a combination of several proposed mechanisms, as well as the possibly of limited biological control of plant pathogens, deserve critical evaluation and reconsideration.

The multitude of options for enhancing plant growth by inoculation with *Azospirillum* led us to propose the ''Multiple Mechanisms Theory,'' based on the assumption that there is no single mechanism involved in promoting plant growth with *Azospirillum*, but rather a combination of a few or many mechanisms in each specific case of inoculation. The mechanisms may vary with the plant species, the strain of *Azospirillum*, and environmental conditions prevailing during the interaction. The effect can be cumulative, as proposed earlier by the ''additive hypothesis'' (Bashan and Levanony, 1990), where the effects of small mechanisms, operating at the same time or consecutively, create a larger final effect on the plant. The effect on plant growth can also be a result from tandem or cascading mechanisms in which one mechanism stimulates the other, which finally yields enhanced plant growth (such as the plausible relations among plant hormones, NO, membrane activities, and proliferation of root). Finally, promoting growth can be the result of a combination of unrelated mechanisms that operate according to environmental or agricultural conditions in a certain location. These include stress mitigation (salt, drought, toxic compounds) and biological control of pathogenic microflora. This inclusive kind of theory may close the gaps between competing theories and might lead to new insights about overlapping and cooperation among seemingly different mechanisms that affect plant growth than have been studied so far.

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