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Physiological and Agronomical Aspects of Phytohormone Production by Model Plant-Growth-Promoting Rhizobacteria (PGPR) Belonging to the Genus *Azospirillum*

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Abstract The functional analysis of phytohormone production, interaction, and regulation in higher plants has re-emerged in the past 10 years due to spectacular advances in integrative study models. However, plants are not axenic in natural conditions and are usually colonized or influenced directly by different microorganisms such as rhizobacteria of which many have the ability to produce phytohormones. This review summarizes information related to the biosynthesis, metabolism, regulation, physiological role, and agronomical impact of phytohormones produced by the model plant-growth-promoting rhizobacteria (PGPR) belonging to the genus *Azospirillum*, considered to be one of the most representative PGPR. We include exhaustive information about the phytohormones auxins, gibberellins, cytokinins, ethylene, and abscisic acid, as well as the plant growth regulators polyamines and nitric oxide. We deal with their metabolism by *Azospirillum* sp. in chemically defined medium, in plant–microbe interactions, or in the context of the agronomical use of *Azospirillum* sp.

Keywords Auxins · Gibberellins · Cytokinins · Abscisic acid · Ethylene · Inoculants

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Rhizosphere and Rhizobacteria

The term rhizosphere is used to describe the portion of soil in which growth of microorganisms is influenced by the presence of the root system (Hartmann and others 2008). The rhizosphere bacteria, so-called rhizobacteria, can be divided into those that form a symbiotic relationship with the plants and those that do not. The latter, called free-living, are closely associated with the root surface or reside within the roots as endophytic bacteria (Kloepper and others 1989). When the presence of rhizobacteria benefits plant growth, those rhizobacteria are called plant-growth-promoting rhizobacteria (PGPR). The vast group of rhizobacteria was divided into different subgroups according to the bacterial promotion mechanisms used during interactions. These subgroups include (1) the PGPR group, proposed by Kloepper and Schroth (1978); (2) the biocontrol-plant-growth-promoting bacteria (biocontrol-PGPB) group, proposed by Bashan and Holguín (1998); and the (3) plant stress homeostasis-regulating rhizobacteria (PSHR) group, proposed by Cassán and others (2009a). However, due to the overlap of these functional subgroups, a clear classification is unrealistic. All PGPR either directly or indirectly facilitate or promote plant growth under nutritional, biotic (biocontrol-PGPB), or abiotic stress conditions. Indirect plant growth promotion induced by biocontrol-PGPB includes a variety of mechanisms, such as rhizosphere competition, induced systemic resistance (ISR), biosynthesis of stress-related phytohormones like jasmonic acid (JA) or ethylene, and biosynthesis of antimicrobial molecules, by which bacteria prevent the deleterious effects of phytopathogens on plant growth. Direct growth-promotion mechanisms induced by PGPR include biological nitrogen fixation, production of phytohormones such as auxins, gibberellins (GAs), cytokinins (CK), and nitric oxide (NO),

iron sequestration by siderophores, and phosphate solubilization. Indirect plant growth promotion and regulation induced by PSHR includes production of stress-related phytohormones or plant growth regulators such as abscisic acid (ABA), JA, cadaverine (Cad), or the ethylene catabolism-related enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase.

Azospirillum sp. as Model of Plant-Growth-Promoting Rhizobacteria

The *Azospirillum* genus is able to colonize more than a hundred plant species and to significantly improve plant growth, development, and productivity under agronomic conditions (Bashan and de Bashan 2010). One of the main mechanisms to explain plant growth promotion of inoculated plants has been related to the ability of *Azospirillum* to produce and metabolize several phytohormones and other plant growth regulatory molecules (Tien and others 1979). As a starting point for this review, we describe the phytohormones in *Azospirillum* sp. that have been studied in the past three decades because (1) a considerable number of molecules have been identified that could be responsible for modifying plant growth and architecture; (2) genes responsible for the synthesis of these compounds and their regulation have been identified; (3) growth responses of inoculated plants have been correlated with the levels of phytohormones measured in the culture medium, rhizosphere, or colonized plant tissues; (4) it has been shown that plant responses to exogenous application of phytohormones can be mimicked by bacterial inoculation; and (5) there is evidence that mutant bacterial strains with altered phytohormone production have different effects on plant hormone balance and growth promotion compared to isogenic strains, and this under a variety of experimental conditions.

Phytohormone Production by *Azospirillum* sp.

Although many mechanisms have been described to explain plant growth promotion by *Azospirillum* sp., a single mechanism is not responsible for the full effect. Multiple mechanisms rather than just one mechanism participate in the association of *Azospirillum* with plants; this hypothesis was suggested 20 years ago as the "additive hypothesis" (Bashan and Levanony 1990; Bashan and de Bashan 2010). One of the main mechanisms proposed to explain the "additive hypothesis" is related to the ability of bacteria to produce or metabolize phytohormones (Tien and others 1979; Okon and Labandera-González 1994). Based on the available data, *Azospirillum* sp. can be linked to the production of several hormonal groups such as auxins (Prinsen and others 1993), gibberellins (GAs) (Bottini and others 1989), cytokinins (CKs) (Tien and others 1979), ethylene (Et) (Strzelczyk and others 1994), abscisic acid (ABA) (Cohen and others 2008), and other plant growth regulators such as nitric oxide (NO) (Creus and others 2005) and polyamines (Cassán and others 2009a).

The following sections contain exhaustive information about the biosynthesis, metabolism, regulation, physiological role, and agronomical impact of phytohormonal compounds produced by *Azospirillum* sp. in chemically defined medium or during plant–microbe interactions.

Auxins

Auxin is the generic name that represents a group of chemical compounds characterized by their ability to induce cell elongation in the subapical region of the stem and to reproduce the physiological effect of the most abundant, naturally occurring auxin molecule, indole-3-acetic acid (IAA). These compounds have been associated with different plant processes such as (a) gravitropism and phototropism, (b) vascular tissue differentiation, (c) apical dominance, (d) lateral and adventitious root initiation, (e) stimulation of cell division, and (f) stem and root elongation (Teale and others 2006).

Auxins and Biological Activity

Several molecules are classified as auxins, but IAA is one of the most prevalent and active in biological systems. Other molecules, including indole-3-butyric acid (IBA) and phenylacetic acid (PAA), in addition to the precursor indole-3-acetonitrile (IAN), are considered active auxins. A variety of inactive molecules, including IAA halogenate compounds such 4-chloroindole-3-acetic acid and conjugated forms with sugars, alcohols, amino acids and glycoproteins, have been identified in plants and bacteria (Glick and others 1999; Korasick and others 2013).

Auxins and *Azospirillum* sp.

Members of the genus *Azospirillum* have provided an excellent experimental model for investigating and understanding the physiological and molecular role of auxins in plants and microbes and also in rhizobacteria–plant interactions. Several naturally occurring auxin-like molecules have been described as products of bacterial metabolism in *Azospirillum* sp. cultures (Fig. 1). In addition to IAA (between 5 and 50 $\mu g\ ml^{-1}$ typically produced according to culture conditions and strain), IBA (Martínez-Morales and others 2003), and PAA (Somers and others 2005), considered in *sensu stricto* as real auxins, many other indolic compounds (precursors and/or catabolites) have been identified in *Azospirillum* sp. supernatants, including indole-3-lactic acid

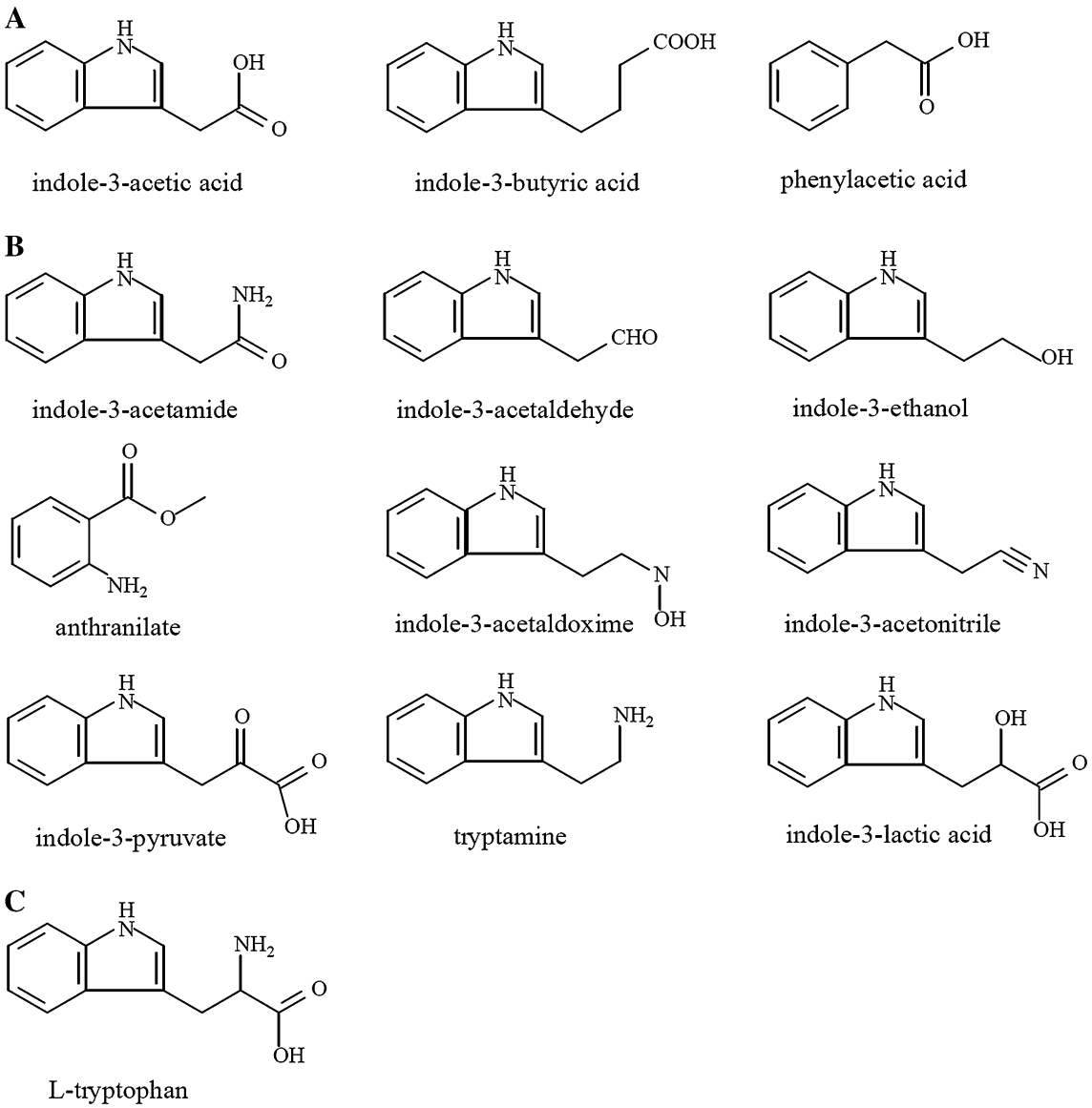

Fig. 1 Chemical structure of the natural auxins (**a**), auxin-like molecules (**b**), and main precursor tryptophan for IAA biosynthesis (**c**) identified and reported in *Azospirillum* sp.

(ILA), indole-3-ethanol and indole-3-methanol (Crozier and others 1988), indole-3-acetamide (IAM) (Hartmann and others 1983), indole-3-acetaldehyde (Costacurta and others 1994), tryptamine (TAM), anthranilate, and other uncharacterized indolic compounds (Hartmann and others 1983). The physiological function of most of these compounds remains unknown, although many of them may serve as precursors or storage compounds for IAA.

IAA and Bacterial Biosynthesis

At least six metabolic routes for IAA biosynthesis have been proposed in bacteria and most of them use tryptophan (Trp) as a precursor. The pathways have been named mostly according to their intermediates such as indole-3-pyruvate (IPyA), indole acetamide (IAM), TAM, and IAN; however, one pathway was named for the key enzyme tryptophan side-chain oxidase (TSO). In addition, a tryptophan-independent pathway has been suggested. Despite this diversity of pathways to produce the active phytohormone, prokaryotic IAA biosynthesis seems to follow predominantly two major routes: the IAM and the IPyA pathways (Lambrecht and others 2000).

Azospirillum sp. and IAA Biosynthesis

Until now, at least four different pathways have been proposed/described for this genus, three Trp-dependent

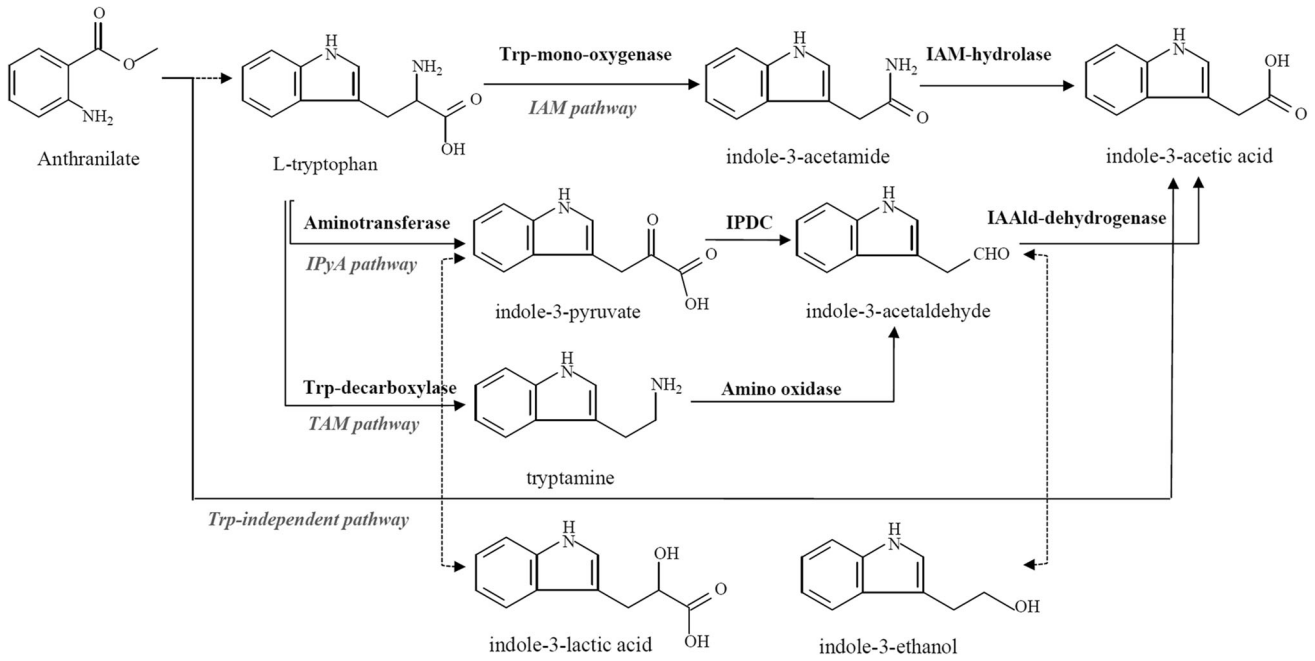

Fig. 2 Hypothetical and characterized pathways to synthesize indole-3-acetic acid (IAA) in *Azospirillum* sp. *Dotted lines* represent hypothetical conversion steps to storage products

pathways (via IPyA, IAM, and TAM) and one Trp-independent pathway (Prinsen and others 1993; Carreño-López and others 2000). See Fig. 2 for an overview of the IAA biosynthesis proposed in *Azospirillum* sp.

Although it has always been suggested that the bacterial IPyA pathway resembles the one present in higher plants (Nonhebel and others 1993), the bacterial and plant IPyA pathways have some differences in the reaction steps downstream from the intermediate IPyA, as demonstrated by recent studies on auxin biosynthesis in plants (Korasick and others 2013). In bacteria, the IPyA pathway starts by converting Trp to IPyA by an aromatic amino acid transferase, followed by decarboxylation by an IPyA decarboxylase (IPDC) to indole-3-acetaldehyde, and concludes with oxidation to IAA by indole-3-acetaldehyde dehydrogenase (Baca and others 1994; Costacurta and others 1994). In this sense, Pedraza and others (2004) detected aromatic aminotransferase (AAT) activity in cell-free crude extracts of four strains of *A. brasilense* and one of *A. lipoferum*, and all strains displayed two isoforms with different molecular weights. Ge and Chen (2009) evaluated the expression and functionality of *atrC* of *A. brasilense* Yu62 in *E. coli* and found that this gene encodes for a novel aminotransferase involved in IAA biosynthesis. Recently, Castro-Guerrero and others (2012) identified the *hisC1* gene in *A. brasilense* Sp7, which encodes aromatic amino acid aminotransferase-1 (AAT1) and they showed that *hisC1* gene expression is induced by root exudates and on plants, suggesting that AAT1 plays a role in conversion of Trp into IAA. The IPyA pathway was initially demonstrated in *A. brasilense* Sp245 by cloning the *ipd*C gene, which encodes an IPDC (Costacurta and others 1994). The expression of this gene is upregulated by the end product IAA itself, which represented the first case of a bacterial gene specifically regulated by auxin (Vande Broek and others 1999). The *ipdC* promoter sequence contains an auxin response element (AuxRE), which is similar to the AuxRE found in gene promoters induced by auxin in higher plants (Lambrecht and others 1999). The IPDC enzyme is the key enzyme in the IPyA pathway because it is the rate-limiting step in this pathway and an *ipdC* knockout mutant is strongly reduced in IAA biosynthesis (Prinsen and others 1993). Certain aspects of the IPyA pathway, such as the characterization and expression regulation of the *ipdC* gene/region, have also been studied in other *A. brasilense* species such as strain Sp7 and SM (Malhotra and Srivastava 2008; Rothballer and others 2005).

In the TAM pathway, Trp is initially converted to TAM, catalyzed by a pyridoxal-phosphate-dependent Trp decarboxylase, followed by conversion to indole-3-acetaldehyde by an amino-oxidase. Although this pathway is present in plants (Conney and Nonhebel 1991) and fungi (Frankenberger and Arshad 1995), it is much less documented in bacteria. This pathway was suggested only for *Bacillus cereus* (Perley and Stowe 1966) and *A. brasilense* (Hartmann and others 1983) based on their ability to produce IAA from TAM in chemically defined culture medium.

Subsequently, Ruckäschel and Klingmüller (1992) detected the intermediates of the pathway in supernatants of *A. lipoferum*, confirming this route in other species of the genus.

The IAM pathway has been studied mainly in phytopathogenic bacteria (Klee and others 1984). The existence of this pathway in *A. brasilense* was suggested by Prinsen and others (1993) and Bar and Okon (1993), who determined the presence of IAM in cell-free supernatant. However, no further work was reported for this pathway in *Azospirillum*.

The Trp-independent pathway has been described through labeled precursor experiments by Prinsen and others (1993), who suggested that conversion of IAA (in the absence of exogenously added Trp) in *A. brasilense* has a distribution of 0.1, 10.0, and 90.0 % for the IAM, IPyA, and Trp-independent pathway, respectively. This latter route has been questioned as attempts to isolate an enzyme or gene responsible for this activity have failed.

Genomic Analysis of IAA Production by *Azospirillum* sp.

To date, six full genomes have been sequenced for bacteria belonging to the genus *Azospirillum*: *A. brasilense* Sp245 (Wisniewski-Dyé and others 2011), *A. brasilense* Az39 (Cassán and others unpublished data), *A. brasilense* CBG497 (Wisniewski-Dyé and others 2012), *A. lipoferum* 4B (Wisniewski-Dyé and others 2011), *Azospirillum* sp. B510 (Kaneko and others 2010), and *A. amazonense* Y2 (Sant'Anna and others 2011). In this section, we aim to unravel putative auxin biosynthesis pathways in *Azospirillum* strains by identifying involved genes in the genome sequence using a BLAST search (Table 1).

A. brasilense Sp245 is one of the most studied strains worldwide and is considered as a typical strain for this species, especially with respect to its auxin biosynthesis. It was isolated from surface-sterilized wheat roots from Paraná state in south Brazil (Baldani and others 1986) and was one of the most promising strains for wheat inoculation in Brazil during the 1980 s. IAA production by *A. brasilense* Sp245 has been studied extensively, with the IPyA pathway the main route for IAA biosynthesis. All genes (aromatic amino transferase, *ipdC*, and aldehyde dehydrogenase) involved in this pathway are present in the genome sequence of *A. brasilense* Sp245. Additionally, two putative nitrilase genes (*NIT1* and *NIT2*) could be identified in Sp245 by p-BLAST, with up to 75 % homology to those studied in *Arabidopsis thaliana* (Wisniewski-Dyé and others 2012). The putative nitrilase enzymes could catalyze the conversion of IAN to IAA via the IAN pathway. However, based on the substrate preference of NIT1 and NIT2 of *A. thaliana* pointing to a role in cyanide detoxification and

Table 1 Presence of putative genes involved in IAA biosynthesis in *Azospirillum* sp.

IAA biosynthesis pathway	IPyA			IAM		IAN	
Enzyme name	Indole-3-pyruvate decarboxylase	Aromatic amino transferase	Aldehyde dehydrogenase	Tryptophan monooxygenase	Indole-3-acetamide hydrolase	Nitrilase	
Gene name	*ipdC*[a]	*hisC1*[b]		*iaaM*	*iaaH*	*NIT1*[c]	*NIT2*[c]
A. brasilense Sp245	+	+	+	–	–	+	+
A. brasilense Az39	+	+	+	–	–	+	+
A. brasilense CBG497	+	+	–	–	–	–	–
A. lipoferum 4B	–	+	–	–		–	–
Azospirillum sp. B510	–	+	–	+ (?)	+ (?)	–	–
A. amazonense Y2	–	–	–	–	–	+	+

The genome sequence of *A. brasilense* Sp245, Az39, and CBG497, *A. lipoferum* 4B, *Azospirillum* sp. B510, and *A. amazonense* Y2 was searched by BLAST for putative genes involved in IAA biosynthesis

[a] *ipdC* from *A. brasilense* Sp245

[b] *hisC1* from *A. brasilense* Sp7

[c] *NIT1* and *NIT2* from *Arabidopsis thaliana*

glucosinolate catabolism, their role in bacterial auxin production can be questioned (Piotrowski 2008).

A. brasilense Az39 has been the most used strain for inoculant formulation in Argentina for more than 40 years. The strain was isolated from surface-sterilized seedlings of wheat plants in the central region of Argentina and selected based on its ability to increase growth and yield of different crops under agronomic conditions (Díaz-Zorita and Fernández Canigia 2009). At the full-genome sequence level, strains Sp245 and Az39 are very similar. Therefore, it is not surprising that all genes encoding for the IPyA pathway can be found in the Az39 genome sequence and are highly similar to the corresponding genes of strain Sp245 (similarity ranging from 97 to 99 %). A gene similar (99 % similarity) to the nitrilase genes of strain Sp245 could also be detected in the genome of Az39.

A. brasilense CBG497 was isolated from maize grown on an alkaline soil in the northeast of Mexico and was able to stimulate maize biomass yield under greenhouse conditions (García-Olivares and others 2007). As for strains Sp245 and Az39, IAA production by *A. brasilense* CBG497 seems to occur via the IPyA pathway. The gene encoding the aromatic amino transferase and *ipdC* involved in this pathway were found in the genome sequence (Wisniewski-Dyé and others 2012). There is no evidence of the presence of nitrilase genes in this strain (Wisniewski-Dyé and others 2012).

A. lipoferum 4B was isolated from the rice rhizosphere (Bally and others 1983) and was successfully used as an inoculant to increase rice yield under field conditions (Charyulu and others 1985). No evidence has been found for the existence of *ipd*C or aldehyde dehydrogenase genes in the genome sequence of *A. lipoferum* 4B. Only a putative aromatic amino transferase sequence with homology to AAT1 from *A. brasilense* Sp7 was identified (Wisniewski-Dyé and others 2012), although it is worth mentioning that these enzymes have a broad substrate spectrum.

Azospirillum sp. B510 is an endophytic bacterium isolated from surface-sterilized stems of rice plants in Kashimadai, Japan (Elbeltagy and others 2001). This strain was not assigned a species name, but based on high homology with the genome sequence of *A. lipoferum* 4B, it can be regarded as a *A. lipoferum* strain. Genome sequence analysis revealed a putative aromatic amino transferase with homology to AAT1 from *A. brasilense* Sp7 (Wisniewski-Dyé and others 2012). Two candidate genes were proposed to be involved in the IAM pathway (Kaneko and others 2010), although we question their role in IAA biosynthesis due to low similarity (especially for the putative *iaaM* gene) with known *iaaM* and *iaaH* genes.

A. amazonense was isolated from forage grasses in the Amazon region, but further studies revealed its broad ecological distribution in association with gramineous plants (Magalhães and others 1983). Although *A. amazonense* is able to synthesize IAA (Rodrigues and others 2008), very little is known about the molecular mechanisms involved (Sant'Anna and others 2011). Genome sequence analysis could not reveal the presence of genes involved in the IPyA or IAM pathway (*ipdC*, *iaaM*, or *iaaH*), but did reveal a gene encoding a protein with about 70 % similarity to nitrilases of *A. thaliana* (Vorwerk and others 2001).

Environmental Factors that Regulate IAA Biosynthesis

The environmental factors that affect IAA biosynthesis in *Azospirillum* sp. are diverse and extensive. Therefore, we discuss only those related to environmental stress and plant signaling (Spaepen and others 2007). The first group of factors includes acidification, osmotic and matrix stresses, and carbon source limitation. The second group includes chemical effectors and molecules produced by plants during stress conditions. IAA production is increased under carbon limitation, during growth rate reduction, and acidic pH (Ona and others 2003, 2005). Interestingly, carbon limitation and growth rate reduction are related to the physiological state of bacteria when entering the stationary growth phase. IAA is produced during all stages of culture growth but increases significantly in the stationary phase (Malhotra and Srivastava 2009). Acidic pH increases *ipdC* gene expression in *A. brasilense*, followed by a subsequent increase in IAA production (Vande Broek and others 2005). Using an *ipdC-gusA* translation fusion, Vande Broek and others (2005) demonstrated that expression of the *ipdC* gene is induced mainly during the stationary growth phase, coinciding with IAA accumulation in the culture medium. IAA production is increased by osmotic stress, abscisic acid (ABA), phytopathogenic fungal effectors, or some L-amino acids when added to exponential cultures of *A. brasilense* strains Sp245 and Az39 (Cassán and others unpublished data). In contrast, oxidative stress, salinity, methyl jasmonate (MeJA), phytopathogenic bacterial effectors, or some L-amino acids decreased IAA accumulation under similar experimental conditions. In other research, the *Pseudomonas fluorescens* F113 secondary metabolite 2,4-diacetylphloroglucinol (DAPG) was proposed as a new environmental signal that induces expression of *A. brasilense* Sp245 genes involved in plant growth promotion (Combes-Meynet and others 2011). The authors showed that both expression of *ipdC* and IAA production are significantly increased by the addition of DAPG in culture medium.

The results summarized in this section suggest that *Azospirillum* sp. strains have the capacity to perceive physiological signals produced (and perceived) by plants or microorganisms under environmental stress conditions and

modify their metabolism to coordinate a unique response together with the plant.

Physiological Effects of Auxins on *Azospirillum* sp.

A microarray analysis to study the overall effects of IAA on the transcriptome of *A. brasilense* Sp245 wild-type and *ipdC* knockout mutant, both cultured in the absence and presence of exogenously added IAA, was reported by Van Puyvelde and others (2011). Based on the multitude of changes observed by comparing the different transcriptomes, the authors concluded that IAA is a signalling molecule in *A. brasilense* allowing the bacterium to adapt itself in the presence of IAA to the plant rhizosphere by changing its arsenal of transport proteins and cell surface proteins.

Physiological and Ecological Effects of Auxins Produced by *Azospirillum* sp.

The primary source of exogenous auxins for plants is the rhizosphere (Patten and Glick 1996). The plant response to exogenous IAA can vary from beneficial to deleterious depending on the concentration perceived by plant tissues and tissue sensitivity. In the case of a beneficial response, the increased hormone content of the rhizosphere due to microbial activity supplements temporarily suboptimal levels in plants and partially modifies the plant cell metabolism with consequent growth promotion (Frankenberger and Arshad 1995). The best reported model of plant growth promotion induced by microbial activity in the rhizosphere is *Azospirillum* sp. for which auxin production has been described to be the main factor responsible for plant growth promotion, based on (1) nodule ontogeny in *Rhizobium*–legume symbiosis, (2) development of the root system in gramineous plants, and (3) regulation of other rhizobacteria.

Effects of Auxins Produced by Azospirillum sp. on Rhizobium–Legume Symbiosis

Most members of the *Rhizobiales* order induce nodule formation on legume roots and these structures provide the plant with fixed atmospheric nitrogen (Bergersen 1971). For over 70 years, since Thiman (1936) proposed that auxins play an important role in the ontogeny (formation and development) of the nodule in *Rhizobium*–legume symbiosis, many studies have indicated that changes in the concentration of this phytohormone or its balance with CK are a prerequisite for nodule organogenesis (Mathesius and others 1997). Therefore, co-inoculation with *Rhizobium* and auxin-producing bacteria can influence the symbiotic outcome by altering the phytohormonal homeostasis.

Inoculation of common bean seedlings with *A. brasilense* resulted in increased production of plant root flavonoids and enhanced capacity to induce *nod* gene expression in *Rhizobium* as compared with noninoculated seedlings (Burdman and others 1996). In many rhizobia the expression of *nod* genes and the synthesis of Nod factors, as well as IAA, are triggered by flavonoids produced by the plant (see Cooper 2007 for a review). The positive effects of *A. brasilense* Cd on legume growth, nodule organogenesis, flavonoids, and lipochitooligosaccharide production were assessed in a *Rhizobium*-common bean hydroponic growth system by Dardanelli and others (2008). Other reports have shown the beneficial effect of co-inoculation with *Rhizobium* and *Azospirillum* in legumes on biological nitrogen fixation, not only at the molecular level or root and nodule morphology, but also for nodule functionality (increase in nitrogenase activity in symbiosomes) (Yahalom and others 1990). Co-inoculation of *Sinorhizobium meliloti* (inefficient IAA producer) with *A. brasilense* (efficient IAA producer) on alfalfa seeds significantly increased the number of root nodules in the primary root. The increase was correlated with the inoculum size. This response could be mimicked by the addition of exogenous IAA (Schmidt and others 1988). Direct evidence of the role of IAA-promoting effects in co-inoculation studies of *A. brasilense* and *R. etli* on common bean was also provided by Remans and others (2008a, b) with the use of the *ipdC* knockout mutant of *A. brasilense*.

Effects of Auxins Produced by Azospirillum sp. on Gramineous Plants

Root growth is perhaps the most remarkably changed parameter in the interaction of PGPR and grasses. The rapid seedling establishment in substrate due to root growth promotion must be considered a clear advantage for plants because it can improve the absorption of water and nutrients. Simultaneously, Tien and others (1979) and Hubbell and others (1979) proved that the exogenous application of IAA, GA_3, and kinetin (a CK) in pearl millet and sorghum produced changes in root morphology similar to those found in seedlings inoculated with *A. brasilense*. Later it was shown that inoculation of *Beta vulgaris* sp. with *A. brasilense* increased the number of lateral roots compared to control plants. This effect was correlated with the high levels of bacterial IAA in pure liquid culture and could be mimicked by exogenous application of similar IAA concentrations (Kolb and Martin 1985). Also, in wheat plants, inoculation with *A. brasilense* simulates the effect of exogenous IAA and GA_3 treatment in regard to the growth pattern of stems and roots (Kucey 1988). Furthermore, the exogenous addition of IAA and nitrate to wheat plants could (partly to fully) be replaced by the inoculation with *A. brasilense* (Zimmer and others 1988). The levels of free IAA and IBA in seedlings of maize (*Zea mays* L.) inoculated with *A. brasilense* Cd were higher than

in noninoculated roots as measured by gas liquid chromatography (GLC) and gas chromatography-mass spectrometry (GC-MS) (Falik and others 1989). Further evidence of the role of bacterial auxin production was provided by inoculation studies with strains altered in IAA biosynthesis: inoculation with a wild-type IAA-producing strain of *A. brasilense* increased the number and length of lateral roots of wheat. In contrast, inoculation with a bacterial mutant with lower IAA production did not modify the root morphology (Barbieri and others 1988). Bothe and others (1992) demonstrated that inoculation of wheat plants with *A. brasilense* significantly increased the formation of lateral roots and slightly increased the dry weight of roots and root hair formation, whereas exogenous application of IAA significantly increased root dry weight but had no effect on the formation of lateral roots. The role of IAA in the phytostimulatory effect upon *Azospirillum* sp. inoculation was demonstrated by comparing the plant-growth-promoting effects of wild-type, auxin-impaired (*ipdC* knockout) mutant, and strains altered in auxin production. Inoculation with the wild-type strain resulted in a decrease in root length but an increase in root hair length and density, whereas inoculation with the *ipdC* mutant did not cause these morphological changes, providing direct evidence for the role of IAA production. In addition, altered IAA production caused by exchanging the native promoter of the *ipdC* gene with a constitutive or plant-inducible promoter further pronounced the effect of inoculation (Dobbelaere and others 1999; Spaepen and others 2008).

Effects of Auxins Produced by Azospirillum sp. on Rhizosphere Microbiota

In regard to the effects of *Azospirillum* sp. on the rhizosphere microflora of inoculated plants, Baudoin and others (2010) evaluated the phytostimulatory effects of *A. brasilense* genetically modified at the level of IAA biosynthesis in rhizosphere microbiota. This study shows that changing the regulation of the *ipdC* in *A. brasilense* can have a significant effect on root-colonizing microbiota. This ecological impact depends not only on the promoter regulating the *ipdC* in genetically modified *Azospirillum* inoculants (that is, constitutive promoter versus root exudate-responsive promoter), but also on the genetic construct itself (that is, presence or absence of a plasmid vector) and the microbial community (that is, bacteria versus fungi).

Gibberellins

Gibberellins (GAs) are a large group of tetracyclic diterpene acids that regulate diverse processes in plants, including germination, stem elongation, flowering, and fruiting (Davies 1995). To date, more than 130 gibberellin molecules produced by plants, fungi, and bacteria have been identified (Hedden and Phillips 2000).

Molecular Structure and Biological Activity

There are two main classes of gibberellins, the free form and conjugated form. Free gibberellins are divided into two subgroups, those that possess the full complement of carbon atoms or C_{20}-GAs and those in which the C_{20} is lost or C_{19}-GAs (Fig. 3). All gibberellins are carboxylated at C_7, with the exception of GA_{12}-aldehyde, and possess one (G_4), two (GA_1), three (GA_8), or four (GA_{32}) hydroxyl functions. The position of the hydroxylation (OH) is very important because it determines the biological activity of the molecule. Hydroxylation of C_3 and C_{13} in their ß and α position, respectively, leads to the activation of the molecule, whereas the hydroxylation of C_2 in position ß has a strong negative effect on activity (Pearce and others 1994). In addition to the free forms, conjugated forms have been identified in plants (Fig. 4). These include glycoside ethers (GA-G), in which a sugar molecule is attached to the structure of the GA by a hydroxyl group, and glycoside esters (GA-GE), in which a sugar residue is bound to the hormone through a carboxyl group on C_7 (Sembder and others 1980). The biochemical and physiological aspects of the GA conjugates have been discussed by Rood and Pharis (1987), who suggest that the main feature of these compounds is the lack of biological activity and the potential reversibility to the active forms by hydrolytic enzyme activity.

Biosynthesis and Metabolism of Gibberellins by *Azospirillum* sp. in Culture Medium

Bottini and others (1989) were the first to confirm the ability of *Azospirillum* sp. to produce gibberellins in chemically defined medium. Using GC-MS analysis, they reported the production of GA_1 and GA_3 in a nitrogen-free medium culture of *A. lipoferum* Op33. Similar results were reported in *A. brasilense* Cd (Janzen and others 1992) and in *A. lipoferum* AZm5 and *A. brasilense* VS9 (Esquivel-Cote and others 2010). In addition, the production of inactive precursors GA_{19} and GA_9 in chemically defined medium of *A. lipoferum* Op33 was reported (Piccoli and Bottini 1996), pointing to the existence of different biosynthetic pathways. The first pathway presumably includes an early 13α-hydroxylation of a common precursor followed by its metabolism to GA_{19} and subsequently to GA_{20}, and finally the 3ß-hydroxylation to GA_1, whereas the second pathway starts with the metabolism of the common

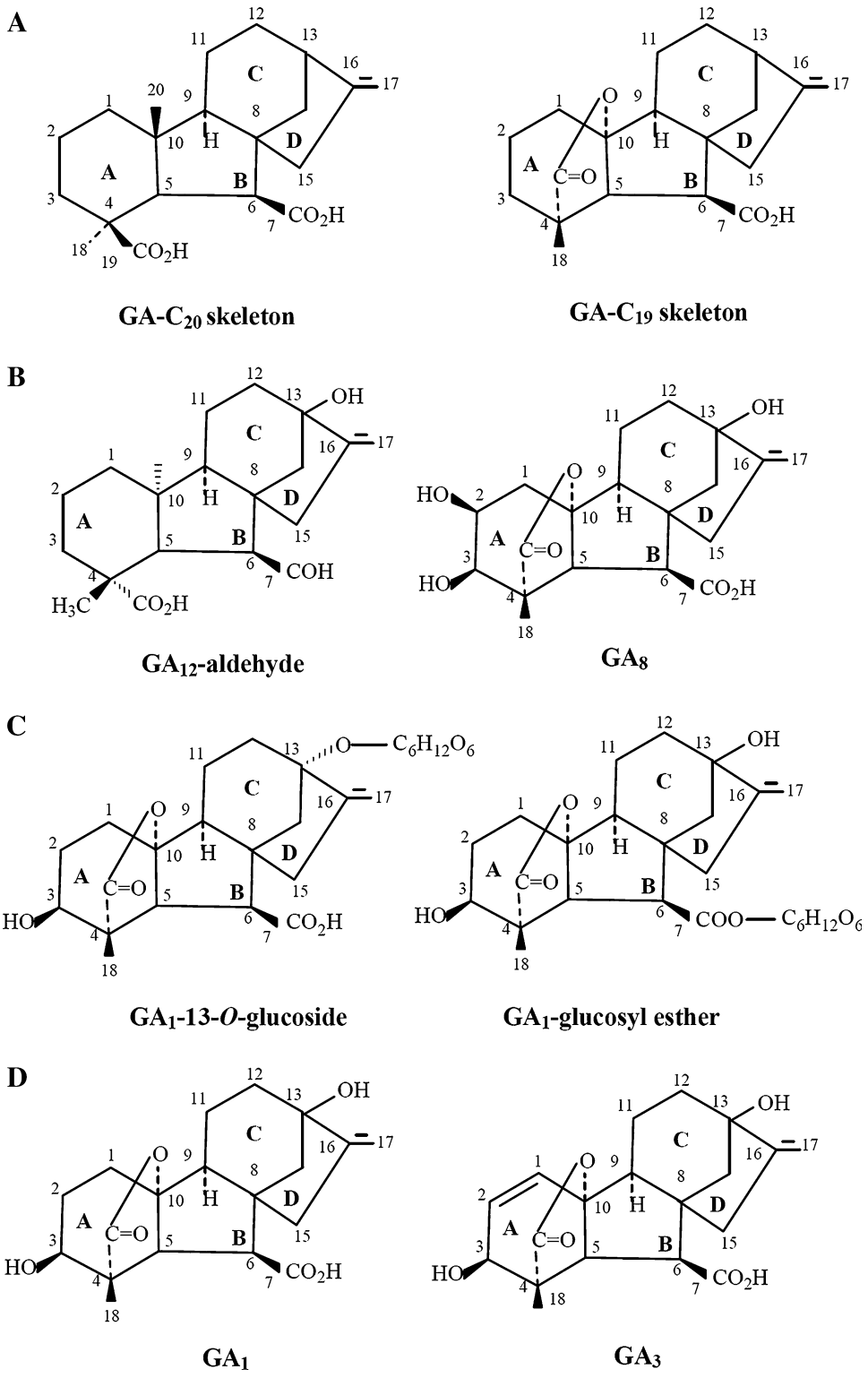

Fig. 3 Chemical structures of gibberellins. **a** Gibberellic C_{20} and C_{19} skeletons. **b** Biologically inactive gibberellins GA_{12}-aldehyde (precursor) and GA_8 (catabolite). **c** Biologically inactive glucosyl conjugates of GA_1. **d** Biologically active GA_3 and GA_1

precursor to GA_9 and the simultaneous 3ß-hydroxylation to GA_4, and finally 13α-hydroxylation to GA_3. These different pathways were confirmed in later reports for *A. lipoferum* Op33 by use of minimal medium supplemented with deuterium-labeled precursors GA_{20} and GA_9 and the identification of deuterated GA_1 and GA_3 by GC-MS (Piccoli and Bottini 1994a, 1996). In addition, *A. lipoferum* Op33 produces GA_{20} and GA_5 in liquid culture medium (Piccoli and Bottini 1996), suggesting the existence of a second 13α-hydroxylation pathway metabolizing GA_{20} to GA_5 and the final 3ß-hydroxylation to GA_3. However, this pathway has not been confirmed. Using labeled precursors,

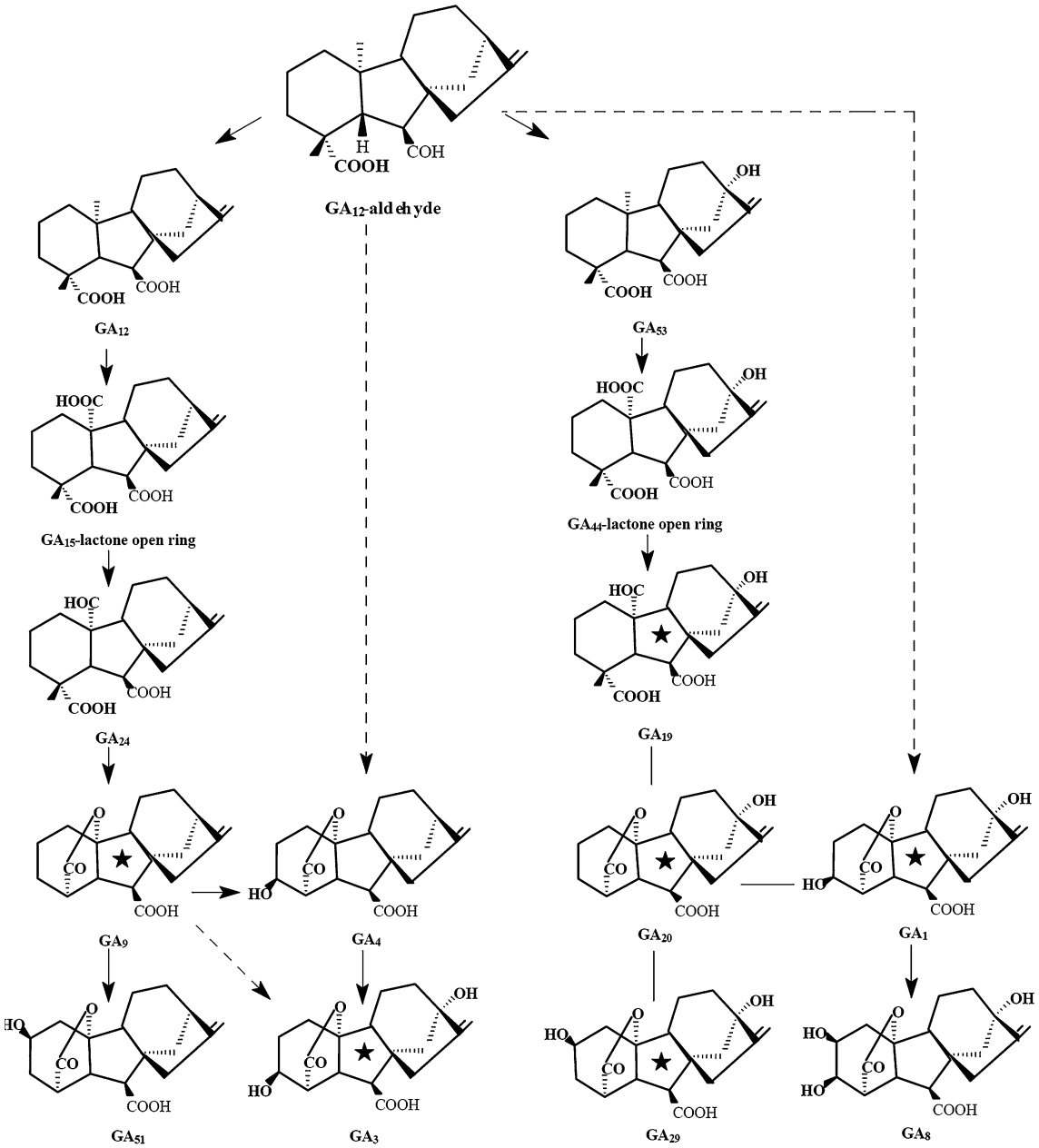

Fig. 4 The putative GA biosynthesis pathways in *Azospirillum* sp. according to the gibberellin molecules identified in the bacteria and the model proposed by Kobayashi and others (1989) in rice (*Oryza sativa* L.). *Dotted arrows* represent reactions in bacteria, *full arrows* represent reactions in plants, and *full lines* represent reactions in both bacteria and plants

similar experiments were performed by Piccoli and others (1997) to evaluate the *A. lipoferum* USDA5b capacity to hydrolyze the gibberellin conjugates with glucose GA_{20}-glycosyl ester and GA_{20}-13-*O*-glucoside and metabolize it to the biologically active molecule GA_1. Production, metabolism, and conjugate hydrolysis of GAs by *Azospirillum* sp. have been comprehensively summarized by Bottini and others (2004).

Biosynthesis and Metabolism of Gibberellins by *Azospirillum* sp. in Planta

The effect of GA-producing bacteria on plants was studied using mostly plant dwarf mutants according to the methods of Murakami (1968) and Kobayashi and others (1989). Seedlings of dwarf cultivars of maize (Phinney and Spray 1988) and rice (Murakami 1972) were inoculated with

A. brasilense or *A. lipoferum* in the presence or absence of plant growth retardants (gibberellin biosynthesis inhibitors) (Rademacher 2000) with the aim of evaluating the reversion of plant dwarfism due to the bacterial biosynthesis and/or metabolism of gibberellins. The first evidence on the capacity of *Azospirillum* sp. to produce active gibberellins *in planta* was reported by Lucangelli and Bottini (1997). They reported the reversal of dwarfism in dwarf mutants of maize and rice by inoculation with *A. lipoferum* USA5b and *A. brasilense* Cd. Cassán and others (2001c) presented evidence about the endophytic capacity of *Azospirillum* sp. to metabolize inactive precursors to active gibberellins by the reversion of rice dwarfism and identification of [17,17-$^2H_2$]-GA_1 by GC-MS in root and shoot tissues pretreated with [17,17-$^2H_2$]-GA_{20}. These results confirmed the capacity of *Azospirillum* sp. to produce GA_1 from GA_{20} through the 3ß-hydroxylation pathway. In similar experimental conditions, Cassán and others (2001a) demonstrated the ability of *Azospirillum* sp. to hydroxylate [17,17-$^2H_2$]-GA_9 to [17,17-$^2H_2$]-GA_3, confirming the existence of a second biosynthetic pathway. Inoculation of rice dwarf mutants pretreated with the early precursor [17,17-$^2H_2$]-GA_{12} also reversed dwarfism. This result can be explained by the ability of bacteria to metabolize $^2H_2$-GA_{12} to the biologically active $^2H_2$-GA_1 or $^2H_2$-GA_3. In regard to metabolism of conjugates, it was observed that *Azospirillum* sp. can reverse genetic dwarfism in inoculated rice seedlings treated with [17,17-$^2H_2$]-GA_{20}-glycosyl ester or [17,17-$^2H_2$]-GA_{20}-glycosyl ether. In these seedlings, phenotypic complementation was observed and the phenotype correlated with the ability of the bacteria (1) to hydrolyze GA_{20}-glucosyl ester or GA_{20}-glucosyl ether to GA_{20} and (2) to metabolize this precursor to the active GA_1 by 3ß-hydroxylase enzymes (Cassán and others 2001b). See Fig. 4 for an overview of the gibberellin biosynthetic pathways proposed in *Azospirillum* sp.

Environmental Factors that Modify GA Production by *Azospirillum* sp.

GA biosynthesis in bacteria increases rapidly at the beginning of the stationary growth phase, suggesting that reduced nutrients in the culture medium may trigger bacterial GA production, as occurs for auxin production (Omay and others 1993). High concentrations of NH_4Cl in the culture medium decreased the amount of GA_3 released (Piccoli and Bottini 1994b), which is comparable to fungi for which gibberellin synthesis initiates when N availability decreases (Rademacher 2000). Other environmental factors are the availability of O_2 and the osmotic potential (Piccoli and others 1999), which can influence the quantity of gibberellins produced by *Azospirillum* sp. The quantity of GA_3 was severely reduced by restricted gas exchange or the addition of PEG as an osmotic agent ($\Psi_w = -1.21$ MPa) in culture medium. This reaction has been considered a compensatory mechanism in the bacterium's ability to produce GA_3 under drought conditions (Piccoli and others 1999).

Physiological Role of GAs Produced by *Azospirillum* sp.

There is an extensive list of publications related to the effects of *Azospirillum* sp. inoculation on growth during the early-development stages in plants, as presented by Bashan and de Bashan (2010). The common changes in plant phenotype upon inoculation can be summarized as follows: (1) increase in root growth, (2) increase in germination rates, and (3) rapid growth of seedlings compared to non-inoculated controls. The increase in root growth can be attributed to mainly bacterial auxin production, whereas the other effects can be attributed, at least partially, to GA production.

Cytokinins

Cytokinins are a group of natural compounds that regulate cell division and differentiation processes in meristematic tissues of higher plants. There are two structural groups of cytokinins: the adenine-type cytokinin group represented by natural and synthetic compounds such as kinetin (K), zeatin (Z), or 6-benzylaminopurine (6-BAP), and the phenylurea-type cytokinin group represented by the synthetic molecules diphenylurea and thidiazuron (TDZ) (Fig. 5). Chemically, adenine-type cytokinins are purines, derived mostly from adenine and modified by substitutions on the N^6, which also include their respective ribotides, ribosides, and glycosides. These plant hormones have been associated with many physiological and cellular processes, including senescence delay by chlorophyll accumulation and organ formation in a wide range of tissues, root development, root hair formation, root elongation, stem initiation, and leaf expansion (Sakakibara 2006). By definition, these compounds (combined with an optimal auxin concentration) induce cell division in plants. The first synthetic cytokinin molecule was discovered by Miller and others (1955) and was named kinetin (K). In 1963, Letham (1963) identified a naturally occurring compound called zeatin (Z) and since then more than 50 molecules and their metabolites have been classified as CKs. The biological activity for all CK-like compounds is not uniform and normally depends on several structural aspects such as a purine ring in the molecule, substitution of N^6 with a simple ribosyl chain isopurine-derived unit, and substitution on positions 2 and 9 of the ring for H, CH_3–S, or an

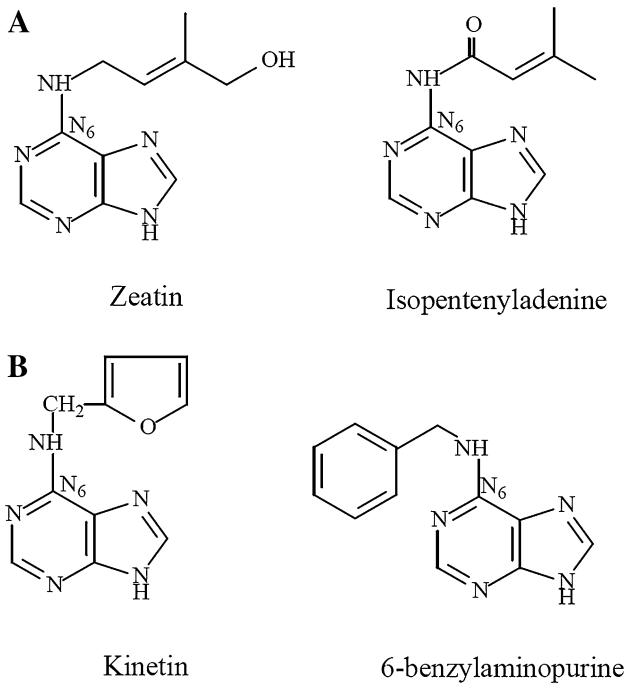

Fig. 5 Structure of cytokinins identified and reported in *Azospirillum* sp. **a** and synthetic molecules **b** with cytokinin activity on plant tissues

unsaturated side chain (optimal five carbons). The natural and synthetic adenine-type cytokinin molecules with confirmed biological activity on plant tissues are zeatin, isopentenyl adenine, kinetin, and 6-benzylaminopurine, and all of them have a double alkyl bridge at position N^6.

Cytokinin Production by *Azospirillum* sp.

At least 90 % of bacteria isolated from the rhizosphere of agriculturally important crops are estimated to be able to produce CK-like compounds in culture medium (Barea and others 1976). Tien and others (1979) were the first to demonstrate the ability of *A. brasilense* to produce CK-like molecules using different types of chromatography (HPLC and TLC) and an inoculation bioassay in pearl millet. However, the partially purified compounds were not fully characterized. Similar results were obtained by Muralidhara and Rai (1986) in *A. lipoferum*. In addition, inoculation caused significant changes in root morphology by increasing the number of lateral roots and the root hair density like those obtained by application of exogenous CKs (Tien and others 1979). A modified chromatography extraction method and application of a radio-immunoassay demonstrated that *A. brasilense* produces isopentenyl adenine, isopentenyl adenine riboside, and zeatin in chemically defined medium (Horemans and others 1986). The lack of information about CK biosynthesis in *Azospirillum* sp. is partially due to the complexity of chemical analysis of these hormones. The most significant work on CK production by *Azospirillum* sp. was published by Strzelczyk and others (1994); they used culture medium supplemented with different C sources. The production of isopentenyl adenine (iP), isopentenyl adenine riboside (iPr), *trans*-zeatin riboside (*trans*-Z), and zeatin (Z) by three strains of *Azospirillum* sp. isolated from the ectomycorrhizal fungi *Rhizopogon vinicolor*, *Laccaria laccata*, and *Hebeloma crustuliniforme* sporocarpus using a soybean callus bioassay was reported by Strzelczyk and others (1994), who used culture medium supplemented with different C sources. However, only the production of iP could be confirmed by GC, which puts into question the production of *trans*-Z and Z. Recently, the production of *trans*-Z by *A. lipoferum* AZm5 growing in defined NFb medium supplemented with NH_4Cl was reported (Esquivel-Cote and others 2010). An interesting case of synergism was described for a mixed culture of *A. brasilense* and *Arthrobacter giacomelloi* which had a higher CK content than that found in the individual cultures of each microorganism (Cacciari and others 1989).

Ethylene

Ethylene (Et) is an important hormone in plant growth and development (Burg 1962). Because of its gaseous state under physiological conditions, for a long time it was not considered a phytohormone, but various studies showed that its synthesis and action are critical for certain physiological processes. Although there are many publications related to the synthesis of this hormone in higher plants, few studies have been published on microbial biosynthesis of ethylene (Arshad and Frankenberger 1993). Et is a simple and symmetrical molecule composed of two carbon atoms (joined by a double bond) and four H atoms. Et is soluble in water and can exert its physiological effects at very low concentrations in plant tissues (almost 0.1 ppm). In higher plants, all tissues have the capacity to synthesize this hormone, but in general Et concentration is associated with the growth state and developmental phase of the plant, with a higher concentration in those tissues involved in active cell division, which are under stressful conditions or in a senescence stage (Burg and Burg 1968).

Biosynthesis and Metabolism of Ethylene by *Azospirillum* sp.

There is little published information on Et production by PGPR and its effect on plant growth. The ability of the free-living bacteria *Azotobacter* sp. and *Bacillus* sp. to produce Et in chemically defined medium was assessed in the early days (Primrose and Dilworth 1976). Later it was shown that *Azospirillum* sp. produces Et in media with

malate, succinate, or pyruvate as a carbon source, reaching a maximal production of 0.17 μmol g^{-1} dry weight when L-methionine is added (Strzelczyk and others 1994).

Not only bacterial production of Et can modify plant growth and hormonal "status"; other hormones (for example, IAA) or bacterial enzymes (for example, ACC deaminase) can alter the level of Et in colonized plants. Tomato seedlings inoculated with the auxin-producing *A. brasilense* strain FT326 showed a positive correlation between IAA concentration, number and length of roots, and plant Et production (up to ten times higher than the controls due to the increase in ACC synthase activity) (Krumpholz and others 2006). This indicates that the plant Et increase is at least partly due to cross-talk between the bacterium-produced IAA and the plant Et biosynthesis, as previously proposed by Rahman and others (2002). The ability of some bacteria to promote plant growth can be correlated to the expression of the bacterial ACC deaminase enzyme (Glick and others 1995). This enzyme can cleave the Et precursor ACC to ammonia and α-ketobutyrate, preventing Et accumulation and its negative effects on plant development. Expression of the ACC deaminase gene *acdS* from *Enterobacter cloacae* in *A. brasilense* resulted in significant improvement in growth of inoculated plants, even better than the results obtained by inoculation with the *Azospirillum* wild-type strain (Holguín and Glick 2001). The ACC deaminase gene *acdS* was detected in some *Azospirillum* strains and several $acdS1^{+}$ strains displayed ACC deaminase activity in vitro (Blaha and others 2006). Recently, it was demonstrated that *A. lipoferum* AZm5 expressing ACC deaminase activity improves the growth of tomato seedlings (Esquivel-Cote and others 2010).

Abscisic Acid

Abscisic acid (ABA) is the plant hormone that is related mainly to homeostatic regulation under abiotic stress conditions (Nambara and Marion-Poll 2005). This hormone confers to higher plants the ability to adapt to stress through a variety of physiological and molecular processes, including osmotic adjustment, stomatal closure, stress-related protein biosynthesis, and gene expression regulation (Davies 1995). From a physiological point of view, ABA supports water economy in plants due to its regulatory effect on stomata and could be considered the true plant signal under salt and drought stress conditions (Zhu 2002).

Abscisic Acid Production by *Azospirillum* sp.

ABA can be produced ubiquitously by higher plants, algae, fungi, and bacteria (Zeevaart 1999). However, there are only a few reports on ABA production by *Azospirillum* sp. in chemically defined culture medium and in inoculated plants. Kolb and Martin (1985) were the first to report on ABA production by *A. brasilense* Ft326 in defined culture medium. However, identification of production was achieved by radio-immunoassay, an insensitive technique compared to mass spectrometry, which is more commonly used today. *A. brasilense* Az39 and Cd have the capacity to produce ABA (75.0 and 6.5 ng ml^{-1} medium, respectively) in chemically defined medium as identified by gas chromatography-mass spectrometry (GC-MS) (Perrig and others 2007). ABA was also characterized by gas chromatography with electron impact mass spectrometry (GC-EIMS) in the supernatant of the model strain *A. brasilense* Sp245 from chemically defined media. When NaCl is added to the culture medium, this strain produces greater amounts of ABA than the control without salt added (235 vs. 73 ng ml^{-1}). Inoculation of *A. thaliana* with *A. brasilense* Sp245 enhances by twofold the plant's ABA content (3.52 ng g^{-1} FW) (Cohen and others 2008). To assay the effects of ABA production on plants, maize plants were treated with fluridone, an inhibitor of plant ABA biosynthesis that results in stunted plants, even in normal watering conditions. Inoculation with *A. lipoferum* USDA 59b completely reversed this effect. The relative water content of fluridone-treated and drought-stressed plants was significantly lower but this effect was neutralized by inoculation with *Azospirillum.* This suggests that ABA produced by *A. lipoferum* contributes to water-stress alleviation (Cohen and others 2009).

Physiological Roles of Abscisic Acid in *Azospirillum* sp.

The role of bacterial ABA in plant–*Azospirillum* interactions is uncertain and there is no direct evidence that this phytohormone promotes or regulates plant growth. However, in restrictive soils (for example, salt- or drought-stressed soils), bacterial ABA could contribute to regulating plant homeostasis and stress response. This is an emerging research line of importance for the PSHR group of PGPRs.

Other Plant Growth Regulator Compounds

Nitric Oxide

Nitric oxide (NO) is a volatile, lipophilic free radical that participates in metabolic, signaling, defense, and developmental pathways in plants (Lamattina and Polacco 2007). NO plays a major role in the IAA signaling pathway and its participation leads to lateral and adventitious root

formation wherein NO acts as an intermediate in IAA-induced root development (Correa-Aragunde and others 2006).

Nitric Oxide Production in Azospirillum sp.

A. brasilense Sp245 and Az39 produce NO in vitro, under anaerobic or aerobic culture conditions (Creus and others 2005; Creus unpublished data). In strain Sp245, NO can probably be synthesized via multiple pathways such as aerobic denitrification and heterotrophic nitrification. NO is produced during the middle and late logarithmic phases of growth (Molina-Favero and others 2007, 2008). NO production in *A. brasilense* Sp245 induces morphological changes in tomato roots regardless of the full bacterial capacity to synthesize IAA. An IAA-attenuated mutant of this strain induces the same physiological changes with slightly less effect on root development as observed for the wild-type strain (Molina-Favero and others 2008).

Polyamines

Polyamines are low-molecular-weight organic compounds with two or more primary amino groups. They are ubiquitous in plant, animal, and microbial cells (Davies 1995). Polyamines serve as growth-regulating compounds in plants and, like phytohormones, when they are present at the appropriate level they display biological activity in processes such as plant growth, development, and stress mitigation (Kuznetsov and others 2006). In this sense, one of the best documented molecules is diamine cadaverine (1,5-diaminopentane), which has been correlated with root growth promotion in pine and soybean (Niemi and others 2001), response to osmotic stress in turnip (Aziz and others 1997), and control of stomatal activity in *Vicia faba* beans (Liu and others 2000).

Polyamine Biosynthesis in Azospirillum sp. and Related Rhizobacteria

Production of putrescine (Put), spermidine (Spd), and spermine (Spm) in chemically defined medium of *Azospirillum* sp. isolated from manioc roots was reported by Thuler and others (2003a). In a second report, Thuler and others (2003b) reported on the production of Put and Spd in chemically defined medium of *Beijerinckia derxii* ICB-10 (ATCC 33962), isolated from the Brazilian savannah. The production of cadaverine (Cad) and other polyamines was reported first by Hamana and others (1988, 1990) for a wide group of α-proteobacteria belonging to the order *Rhizobiales*, but recently proposed for *A. brasilense* by Cassán and others (2009a). Similar results were reported by Goris and others (1998) in an extensive group of strains belonging to the so-called *Pseudomonas* rRNA group I (the authentic pseudomonads) and *Azotobacteraceae* (free-living nitrogen-fixers). All evaluated strains showed Put, Spd, and Cad production capacity, although for some authentic pseudomonads, Cad could not be identified.

Physiological Role of Polyamines Produced by Azospirillum sp.

The diamine Cad has been correlated with root growth promotion (Gamarnik and Frydman 1991; Niemi and others 2001) and osmotic stress mitigation (Aziz and others 1997; Liu and others 2000) in plants. *A. brasilense* Az39 has the capability to produce Cad in chemically defined medium and as an endophyte in rice seedlings. This capacity is correlated with root growth promotion and osmotic stress mitigation under hydroponic culture conditions. Therefore, bacterial Cad production was proposed as a novel bacterial mechanism involved in plant growth promotion and/or regulation of the plant response to osmotic stress (Cassán and others 2009a).

Crosstalk of Bacterial Hormone Production with Plant Phytohormones

Biochemical, molecular, physiological, and functional analyses of phytohormone interactions in higher plants have re-emerged in the last 10 years, and because of this revival, the bacterial phytohormones are not exempt from the same analyses. The "crosstalk" interactions described in the literature for several plant species may give new insights into the simple phytohormonal growth promotion-dependent model described for *Azospirillum* sp. In this regard, there is circumstantial evidence of the interaction between the phytohormones produced by *Azospirillum* sp. and the hormonal background of inoculated plants. Moreover, a detailed analysis of this interaction may reveal specific interactions that could result in a PGP effect. In this regard, Fulchieri and others (1993) found that maize seedlings inoculated with three *A. lipoferum* strains showed significantly improved root and shoot growth compared to seedlings inoculated with a single strain. In these trials GA_3 was identified in the free acid fraction of plant extracts and these results led speculation about the bacteria's ability to increase the in vivo pool of biologically active GAs in the roots of inoculated plants. It was shown later that auxin could promote, at least in part, stem elongation by increasing endogenous levels of 3β-hydroxylated GAs (Ross and O'Neill 2001), which could directly be related to the results of Fulchieri and others (1993). Thus, part of the growth response observed in inoculated plants might be the result of the bacterial production of GAs or the plant

production of GAs induced by bacterial IAA (Yaxley and others 2001; Ford and others 2002; Inada and Shimmen 2000). However, it is very difficult to discriminate between different phytohormone pools and therefore careful analyses are necessary to pinpoint the changes in phytohormone levels due to inoculation.

Another case of crosstalk is the study of the growth response of tomato seedlings inoculated with *A. brasilense* FT326, correlating plant phenotypes and Et production. The increase in Et production was accompanied by increased activity of ACC synthase in plant tissues (Krumpholz and others 2006). These results indicate that the Et increase is in part due to crosstalk between the bacterially produced IAA and Et synthesis by the plant (Rahman and others 2002).

Although a number of interactions between bacterial and plant phytohormones were discussed in this section, the ability of bacteria to modify the plant's balance between auxin and CKs (due to the production/degradation of IAA and/or CKs) is worth mentioning because this balance determines the root/shoot ratio. However, no in-depth studies on this balance have been published, except a detailed analysis on the auxin/cytokinin production by soil- and plant-associated bacteria (Van Laer 2003).

Azospirillum-Based Inoculants and Plant Growth Promotion

A flourishing inoculant business has developed in South America, possibly due to the large area dedicated to extensive agriculture and the reproducible results under field conditions. This section focuses on the Argentina experience as a case study of the level of agronomic use of *Azospirillum*-based inoculants.

From 1981 to 1996, the Instituto de Microbiología y Zoología Agrícola (IMYZA), INTA-Castelar from Argentina, developed an intensive program with the main objective of selecting and identifying *Azospirillum* strains and to evaluate their ability to promote plant growth in different crop species. The experiments showed that there was a more pronounced effect with *A. brasilense* than with *A. lipoferum* on most evaluated plant species, and they showed that *A. brasilense* Az39 was the most promising strain for inoculant formulation based on its ability to increase growth and yield of evaluated crops in the range of 13–33 %. Based on this information, the National Agricultural Health Service (SENASA) proclaimed a nationwide recommendation of the native strain *A. brasilense* Az39 for inoculant production for use on maize, wheat, and other nonlegume plant species. From a physiological point of view, the plant-growth-promoting capacity of *A. brasilense* Az39 has been confirmed agronomically by its effectiveness for the past 30 years in increasing the productivity of inoculated crops in a large number of assays under field conditions (Díaz-Zorita and Fernández Canigia 2009). However, a detailed description of the main plant-growth-promoting mechanisms of this strain was lacking. The Laboratorio de Fisiología Vegetal y de la Interacción Planta-microorganismo of the Universidad Nacional de Río Cuarto (Argentina) has elucidated the potential mechanisms responsible for the growth promotion by this strain. As part of these results, Perrig and others (2007) showed that *A. brasilense* Az39 has the ability to produce and release IAA, Z, GA_3, ABA, and Et in vitro. Strain Az39, used an inoculant alone or in combination with *B. japonicum* E109, has the capacity to promote seed germination and early growth in soybean, wheat, and maize (Cassán and others 2009b). This strain was also able to produce and release GA_3, Z, and IAA in culture medium in sufficient concentrations to produce morphological and physiological changes in treated seeds or seedlings. The concentrations of GA_3, Z, and IAA increase during the stationary growth phase because bacteria produce phytohormones during the exponential and early stationary growth phase, but these molecules are continuously accumulated in the culture medium because of the "batch fermentation model." Finally, the accumulation of phytohormones alters the capacity of the bacterial culture (inoculant) to promote seed or seedling growth, and because this effect is not strictly dependent on the bacterial cell, it could be defined as the "hormonal effect of inoculation" and might be extended to other phytohormone-producing PGPR.

Conclusion and Future Prospects

Phytohormone production by bacteria has been a research topic for many decades, first in pathogenic (for example, *A. tumefaciens*) and later also in beneficial plant-associated bacteria. Relatively little is known about phytohormones in *Azospirillum* sp. and other bacteria as compared to model plants. Aspects that are well characterized are the biosynthesis pathways involved in phytohormone production, although for many phytohormones we need to rely on plant data to fully understand the bacterial biosynthesis. A prerequisite to estimate the importance of bacterial phytohormone production in plant growth promotion is the use of mutants impaired in production, for example, a knockout mutant missing a key biosynthetic gene. Only in the case of auxins (particularly for IAA) has the physiological and molecular functionality in both chemically defined medium and plant–microbe interaction been described. For gibberellins, and particularly for gibberellic acid and GA_1, the model has been described from a physiological but not from a molecular point of view. Finally, for CKs, ABA, Et, polyamines, and NO, the ability of several microorganisms to produce these compounds in chemically defined medium

Table 2 Overview of plant growth regulators produced in vitro by *Azospirillum* sp.

Class[a]	Hierarchy[b]	Molecules	References
Auxins	1st	IAA, PAA, IBA	Prinsen and others (1993) Martínez-Morales and others (2003) Somers and others (2005)
Gibberellins	4th	GA_3, GA_1, GA_9, GA_{20}, GA_{19}, GA_5	Bottini and others (1989) Piccoli and Bottini (1996)
Cytokinins	3rd	iP, iPr, Z, *t*-Zr	Horemans and others (1986) Esquivel-Cote and others (2010)
Ethylene	5th	Et	Strzelczyk and others (1994)
Abscisic acid	6th	ABA	Kolb and Martin (1985)
Nitric oxide	2nd	NO	Creus and others (2005)
Polyamines	7th	Cad, Spm, Spd, Put	Cassán and others (2009a) Thuler and others (2003a)

IAA indole-3-acetic acid, *PAA* phenylacetic acid, *IBA* indole-3-butyric acid, *NO* nitric oxide, *iP* isopentenyl adenine, *iPr* isopentenyl adenine riboside, *Z* zeatin, t-Zr *trans* zeatin riboside, $GA_{3,1,9,20,19}$ gibberellins n, *Et* ethylene, *ABA* abscisic acid, *Cad* cadaverine, *Spm* spermine, *Spd* spermidine, *Put* putrescine

[a] Molecules identified from *Azospirillum* sp. liquid cultures by precise methodology (HPLC, GC-MS)

[b] Related to the importance of the phytohormonal role in its interaction with the plant considered by the authors based on available evidence

has been demonstrated but not fully understood in plant–microbe interactions (see Table 2 for an overview).

Integration of both microbial and plant models from a phytohormone and physiological point of view could be the beginning of a better understanding of the plant–microbe interactions into a new concept named "plant–microbe physiology" or simply "integrative physiology." As we described throughout this review, *Azospirillum* sp. have the capacity to modify growth, development, and behavior of several plants, even in stress conditions. However, molecular evidence at the level of plant–hormoneplant–microbe interaction is lacking and this level is certainly a domain emerging for study in the future.

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