

Assessment of affinity and specificity of *Azospirillum* for plants

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Abstract

Background *Azospirillum* spp. are the most studied plant growth-promoting bacteria (PGPB). The genus represents a common model for plant-bacteria interactions. This genus was initially isolated and tested on cereals and was subsequently commercialized.

Dedication This study is dedicated to the memory of the Israeli soil microbiologist Prof. Yigal Henis (1926–2010) of the Faculty of Agriculture, The Hebrew University of Jerusalem in Rehovot, Israel, one of the pioneers of research of *Azospirillum*.

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Aims Despite claims of plant specificity, particularly towards cereals, data over the past 40 years does not appear to substantiate claims of such specificity/affinity of *Azospirillum* species. Consequently an evaluation of the specificity/affinity of the genus *Azospirillum* across all plants, in general, and cereals, in particular, was undertaken.

Results Although the majority of studies focused on cereals, *Azospirillum* spp. increase growth of 113 plant species across 35 botanical families, including 14 species of cereals. Amongst *Azospirillum* spp., several well studied strains have been effective in several plant species, making these organisms potentially valuable for further study.

Conclusions This review demonstrates that azospirilla are not cereal-specific at the genus and species levels. *Azospirillum* serves as a general PGPB to every plant species tested so far. Given the paucity of widespread screening, affinity of strains to a plant genotype, cultivar, or plant species cannot be overruled. Definitive conclusions concerning such specificity require molecular and cross-inoculation studies, using various strains of bacteria, and re-isolation after growth of the plants in different plant species. (203 words).

Keywords *Azospirillum* · Attachment · Colonization · Affinity · Specificity

Introduction

Bacteria of the genus *Azospirillum* possess numerous properties allowing them to survive and thrive in the

nutrient rich and protective environment that exists in the rhizosphere of plants (Steenhoudt and Vanderleyden 2000). With the advantage of multiple plant growth promoting mechanisms (Bashan and de-Bashan 2010), *Azospirillum* strains have been tested and used as inoculants in crop production, initially with cereals, but later with other plants.

When the genus *Azospirillum* (Beijerinck 1925) was re-discovered by the late Johana Döbereiner and her colleagues in Brazil in the 1970s it was heralded as an associative plant growth-promoter for cereals (Döbereiner and Day 1976). Consequently, initial studies regarding the agronomic potential of these plant growth-promoting bacteria (PGPB) were conducted exclusively on cereals, as is often still the case (for reviews: Bashan and Levanony 1990; Okon and Labandera-Gonzalez 1994; Bashan and Dubrovsky 1996; Dobbelaere et al. 2001; Bashan et al. 2004; Massena-Reis et al. 2011, Table 1). The wealth of data concerning its interaction with cereals led to the conclusion that *Azospirillum sp.* has some as yet undefined specificity for gramineous plants (Baldani and Döbereiner 1980). By comparison to the specificity of *Rhizobium*-legume symbiosis, which is evident at early stages of the infection and involves specific molecular signaling among the bacteria and their host (Lerouge et al. 1990), caution is required when considering potential specificity of *Azospirillum*-cereal interactions, as the evidence better supports a possible affinity of some strains for cereals, rather than any specificity (Drogue et al. 2012).

In subsequent studies *Azospirillum* species and strains known to affect the growth of cereals have been also tested on other species of plants worldwide. It has since become clear that many isolates of this genus can improve the growth and influence the metabolism of many plant species across many families, including annuals, perennials, trees, ornamental, spices, wild plants and even single cell microalgae (for documented examples see Table 1 and Table S1). Consequently, it is clear that *Azospirillum* can interact with a wide variety of plants and its species serve as a general plant growth-promoting bacteria (PGPB). However, the question still remains whether genus/species affinity for specific plants is evident and whether host specificity exists.

Given the pervasive contention that cereal-specificity of *Azospirillum* exists, critical re-evaluation of published research over the past four decades of implied specificity or preferred affinity of *Azospirillum* is

essential. This review was driven by comparing four hypotheses derived and proposed from the prevailing assumptions within the literature: (1) *Azospirillum* as a genus has inherently a higher affinity for cereals and the effects recorded on other plant species are the exception, (2) *Azospirillum* has higher affinity to certain plant species but the increased affinity is at the species or strain level, (3) *Azospirillum* strains colonize and use a narrow range of plants as hosts, thus demonstrate host specificity and (4) *Azospirillum* species/strains are non-specific plant growth promoting bacteria affecting the metabolism of plants in general and some species/strain have a wide host range.

To examine these hypotheses we evaluated the literature using the following criteria: (1) the variety of plant species that show response to *Azospirillum* inoculation in general, (2) evidence for specific/unique reaction of plant only to certain *Azospirillum* species or strain (3) plants that have been shown to be colonized by *Azospirillum* and whether the attachment was exclusively by particular species/strains and (4) any knowledge of species/strain specific properties, including molecular traits, that are related to *Azospirillum* attachment to plants. Consequently, this essay was organized, as follows: historic background of the topic, phenomena of interaction of common strains of *A. brasilense* with multiple hosts, attachment and initial colonization as parameters of potential affinity/specificity, and molecular studies providing potential indicators of affinity of strains to plants.

The historic theme of “Specificity” of *Azospirillum*

The genus *Azospirillum* has currently 12 species (Lavrinenko et al. 2010), with the most studied species including: *A. brasilense*, *A. lipoferum* (Tarrand et al. 1978), *A. halopraeferens* (Reinhold et al. 1987), and *A. oryzae* (Xie and Yokota 2005). Recently, two species *A. amazonense* (Magalhães et al. 1983) and *A. irakense* (Khammas et al. 1989) were re-classified as *Niveispirillum irakense* and *Nitrospirillum amazonense* (Lin et al. 2014). Early claims of *Azospirillum* specific affinity for certain cereal species (Bashan and Levanony 1990) relied on inoculation studies that focused on strains of *A. brasilense* and *A. lipoferum* and were based on the following cases: (1) When responses of C₃ and C₄ plants were tested, *A. lipoferum* predominantly colonized C₄ plants while *A. brasilense* predominantly

associated with C_3 plants both in tropical (Baldani and Döbereiner 1980; Baldani et al. 1986) and in temperate zones (Haahtela et al. 1981; Lamm and Neyra 1981). (2) Enhanced performances of cereal plants were more frequent when specific plant-bacterial species combination was used (Baldani et al. 1983, 1987; Reynders and Vlassak 1982; Pereira et al. 1988).

Moreover, strains of *A. brasilense* (SpT60, JM6A2 and Cd), isolated from different plants: wheat (*Triticum* spp.), maize (*Zea mays*), and Bermuda grass (*Cynodon dactylon* (L.) Pers.), respectively, showed distinct chemotactic response to organic acids, which correlated with the exudates of the respective plants of origin (Reinhold et al. 1985). Specificity can occur at the plant cultivar level; only a few of many tested cultivars of the same plants responded to inoculation with a given strain of *Azospirillum* (Bouton et al. 1979; Wani et al. 1985; Millet et al. 1986; Walker et al. 2011; Chamam et al. 2013), and plant genotype affected the response to *Azospirillum* inoculation (Garcia de Salamone and Döbereiner 1996; Garcia de Salamone et al. 1996).

The proposal of host specificity of *Azospirillum* was reinforced by discoveries in molecular biology. Finding sequences on *Azospirillum* plasmids with homology to rhizobial nodulation genes *nodPQ* and *nodG* during the late 1980s to early 1990s (Vieille and Elmerich 1990) supported the contention that specificity existed, arguing for an ability of *Azospirillum* to nodulate certain hosts. However, further analysis demonstrated no involvement of *nod* genes in *Azospirillum*-root interactions (Vieille and Elmerich 1992). Use of fluorescent probes demonstrated that the very common *A. brasilense* strain Sp245 is an internal root colonizer (Schloter et al. 1994), whereas the common strain Sp7 only colonized the root surface. Yet, this might differ from variety to variety or species to species. Contemporaneous research attempted to facilitate the interaction using synthetic auxins to create paronodules on cereal roots that were colonized by *Azospirillum* (Tchan et al. 1991; Christiansen-Weniger 1992; Kennedy and Tchan 1992; Zeman et al. 1992; Sriskandarajah et al. 1993; Yu et al. 1993; Christiansen-Weniger and Vanderleyden 1994; Katupitiya et al. 1995a, b; Kennedy et al. 1997). Although this procedure did not result in endophytic colonization by strain Sp7 or establish a long-term colonization with other species, this approach created a new dimension in N_2 -fixation, which unfortunately was not developed further.

While the vast majority of publications have been on *Azospirillum*-cereal interaction, the proposal that the genus *Azospirillum* exclusively or mainly enhances growth of cereals has been confronted with newer evidence demonstrating effect on numerous other plant species from a variety of families (Table 1, Table S1). Currently, *Azospirillum* species are known to positively affect 113 plant species of which 14 are cereals and the rest non-cereals (Fig. 1a) for 34 additional botanical families (Fig. 1b).

The accumulative data of the last three decades indicates that *Azospirillum* as a genus has the ability to interact with a wide variety of plants, including crop plants, weeds, annuals and perennials, and can be successfully applied to plants that have no previous history of *Azospirillum* in their roots. It appears that *Azospirillum* is a general rhizosphere colonizer and a general plant growth-promoter and its interaction with plants does not resemble legume-rhizobia specific interactions. This data does not preclude the possibility that *Azospirillum* species and strains may demonstrate plant preference, a possibility that must be investigated.

Interaction of *Azospirillum* strains with multiple host plants

Only a small variety of strains, including strains of *A. brasilense* and *A. lipoferum* have been commonly used in inoculation trials, some of which are commercially available for a variety of crops. Several examples of strains can demonstrate the multifaceted of activity on plants.

One of the best examples of a relatively promiscuous isolate is *A. brasilense* Cd/Sp7. *A. brasilense* Cd was isolated from plants inoculated with strain Sp7, thus they were sometimes considered to be one strain; however they have been known to display different phenotypes. They constitute one of the most studied strains for *A. brasilense*, having been isolated originally from a gramineous weed (*Cynodon dactylon*, Eskew et al. 1977) and commonly used as a reference strain. Initially, this strain was shown to colonize and enhance the growth and the yield of many winter and spring cereals (Kapulnik et al. 1981, 1983; Lin et al. 1983; Smith et al. 1984; Yahalom et al. 1984; Bashan 1986a; Assmus et al. 1995) and to move in soil towards wheat plants (Bashan 1986b; Bashan and Levanony 1987; Bashan and Holguin 1994). It had a marked capacity

Table 1 A sampler of the effect of inoculation of species of *Azospirillum* spp. on different plant species

Type of plant	Plant species	bacterial species**	Test conditions	Effect on plant	Range of years of studies	country	References
Graminaceae	Numerous cereals	Ab, AI, A.sp	Greenhouse and field	Enhanced growth and mineral uptake	1976–2004	Brazil, Israel, USA, France, Mexico, Argentina	For reviews: Bashan and Levanyon 1987; Okon and Labandera-Gonzalez 1994; Dobbelaere et al. 2001; Bashan et al. 2004; Belimov et al. 2004
Weeds	Wilman grass (<i>Eragrostis superba</i> Peir.), Weeping lovegrass (<i>Eragrostis curvula</i> (Schrad.) Ness), Sideoats grama (<i>Bouteloua curtipendula</i> (Michx.) Torr.)	Ab	Greenhouse	Increase in biomass	2004	Mexico	Arias et al. 2004
	Pearl millet (<i>Pennisetum glaucum</i> (L.) R.Br.)	AI Az204 mixed with <i>Rhizobium</i>	Pot culture	Increase in germination, seedling vigor, plant height and seed weight	2005	Korea	Poongguzhali et al. 2005
	48 species	Ab	Field	Root colonization	1987, 1995	Israel	Bashan and Levanyon 1987*; Bashan and Holguin 1995*
Vegetables	Tomato (<i>Solanum lycopersicum</i> L.)	AI, Ab	Greenhouse	Enhanced growth parameters; protection against pathogens	1989	Israel	Bashan et al. 1989c*; 1991*; Levanyon and Bashan 1991*; Bashan 1998*; Bashan and Vazquez 2000*; Bashan and de-Bashan 2002a*, b*
	Tomato	A.sp	Greenhouse	Enhanced growth parameters; protection against pathogens	1995–2004	India	Gupta et al. 1995*; Siddiqui 2004*
	Tomato	Ab Cd	Growth chamber and greenhouse	Enhances root length Increases number of lateral roots and root hairs	2013	Italy	Botta et al. 2013*
	Tomato	A.sp, Ab	Shadehouse	Increase in biomass Enhanced several growth parameters	2003	Argentina	Romero et al. 2003*
	Tomato	Ab	In vitro and pots	Enhanced several growth parameters	1987	Israel	Hadas and Okon 1987*
	Tomato	Ab	In vitro and growth chamber	Enhanced lateral root and root hair development; change in rhizosphere community; antifungal activity mitigation of water stress	2004–2008	Argentina Italy and Germany	Becker et al. 2002*; Grilli-Caiola et al. 2004*; Creus et al. 2005*; Ribaudo et al. 2006*; Correa et al. 2007*; Felici et al. 2008*; Molina-Favero et al. 2008*; Romero et al. 2014
	Tomato	AI, Ab	Pot culture	Improved N uptake	2010	Mexico	Esquivel-Cote et al. 2010
	Tomato	Ab CW903	Greenhouse	Improved nutrient uptake, Enhance plant growth	2010	Korea	Madhaiyan et al. 2010*
	Tomato	Ab	In vitro	Enhance root length	2001	Canada	Holguin and Glick 2001, 2003*
	Eggplant (<i>Solanum melongena</i> L.)	Ab	Greenhouse	Enhanced growth parameters	1989	Israel	Bashan et al. 1989b*; Bashan 1998*
	Pepper (<i>Capiscum annum</i> L.)	Ab	Greenhouse	Enhanced growth parameters	1989	Israel	Bashan et al. 1989b*; 1991*; Bashan 1998*

Table 1 (continued)

Type of plant	Plant species	bacterial species**	Test conditions	Effect on plant	Range of years of studies	country	References
Pepper	Pepper	Ab	Greenhouse	Improved nutrient uptake, Enhance plant growth	2010	Korea	Madhaiyan et al. 2010*
	Pepper	Ab	In vitro, greenhouse and commercial greenhouse	Effect on fruit parameters; Improve nitrogen in plants; Increased in flavonoids and anthocyanins	2008, 2009, 2010, 2011	Spain	del Amor et al. 2008; del Amor and Porras 2009; Flores et al. 2010; del Amor and Cuadra-Crespo 2011
Ornamental/spices/forage	Black pepper (<i>Piper nigrum</i> L.)	A.sp	Nursery	Improve plant parameters and cuttings	1989–2000	India	Govindan and Chandry 1985
	Cucumber (<i>Cucumis sativus</i> L.)	Ab Sp245	Hydroponic	Longer hypocotyl, increase in root projected area	2010	Argentina	Pereyra et al. 2010*
	Lettuce (<i>Lactuca sativa</i> L.)	Ab	Growth chamber	Higher germination, Increase in biomass	2006	Argentina	Barassi et al. 2006*
	Fennel (<i>Foeniculum vulgare</i> Mill.)	Al mixed with <i>Azotobacter chroococcum</i> y <i>B. megatherium</i>	Field	Increase vegetative growth	2007	Egypt	Mahfouz and Sharaf-El-din 2007
	Sweet potato (<i>Ipomoea batatas</i> (L.) Lam)	Ab	Field	Lower foliage yields	1990	USA	Mortley and Hill 1990
	Mustard (<i>Brassica juncea</i> (L.) Coss.)	Al	Field	Increase N uptake; growth promotion	1985	India	Saha et al. 1985*; Kesava Rao et al. 1990; Gamo and Ahm 1991
	Blanket flower (<i>Gaillardia pulchella</i> Fong)	A.sp	Field	Several plant parameters	2004	India	Gadagi et al. 2004
	Alfalfa (<i>Medicago sativa</i> L.)	Ab	Greenhouse	Increase shoot growth, nodule number and surface area	1993	Israel	Itzigsohn et al. 1993
	Burr medic (<i>Medicago polymorpha</i> L.)	Ab	In vitro	Increase number of nodules	1990	Israel	Yahalom et al. 1990
	Clover (<i>Trifolium</i> sp. L.)	A.spp	In vitro	Stimulation of nodulation	1985	Australia	Pizzinski and Rolfe 1985
Industrial crops	Marigold (<i>Tagetes minuta</i> L.)	AbSp7 coinoculated with <i>P. fluorescense</i>	Greenhouse	Increase in root dry weight, increase in total phenol content	2013	Argentina	Cappellari et al. 2013*
	<i>Phytinia</i> sp. Lindl.	Ab Cd4, Ab Sp7	Growth chamber	Thicker cuticle	2010	Argentina	Larraburu et al. 2010
	Strawberry (<i>Fragaria ananassa</i> (Weston) Duchesne)	Ab Sp7	Growth chamber	Biocontrol of <i>Colletotrichum</i> in strawberry	2011	Argentina	Tortora et al. 2011a, b
	Sunflower (<i>Helianthus annuus</i> L.)	Al	Greenhouse	Enhanced growth	1991	France	Fages and Arsac 1991
	Sunflower	Ab	In vitro	Enhanced colonization	2010	India	Joe et al. 2010*
	Cotton (<i>Gossypium hirsutum</i> L.)	Ab	Greenhouse	Enhanced plant dry weight and nitrogen uptake	1987	Egypt	Fayez and Daw 1987*
	Cotton	Ab	Greenhouse	Enhanced growth parameters	1989	Israel	Bashan et al. 1989b*
	Cotton	Ab	Greenhouse	Plant protection from herbicide	1997	Australia	Feng and Kennedy 1997
	Oil palm (<i>Elaeis guineensis</i> Jacq.)	Ab, Al	Glass house, field	Improve N uptake, photosynthesis and better growth	2001	Malaysia	Amir et al. 2001, 2005
	Palmarosa (<i>Cymbopogon martinii</i> (Roxb.) Wats.)	Ab	Glasshouse	Enhance growth and oil content	1996	India	Ratti and Janardhanan 1996
Canola (<i>Brassica napus</i> L.)	Ab	Field		2011	Pakistan	Nosheen et al. 2011	

Table 1 (continued)

Type of plant	Plant species	bacterial species**	Test conditions	Effect on plant	Range of years of studies	country	References
Non-cereal grains	Flax (<i>Linum usitatissimum</i> L.)	Ab B-4485	Field	Increase in leaf protein and chlorophyll content. Increase in seed protein	2006	Belarus	Mikhailouskaya 2006*
	Bean (<i>Phaseolus vulgaris</i> L.)	Ab	Growth chamber	Enhanced growth parameters	2000	Israel	German et al. 2000
	Bean	Ab	In vitro in pots	Increase nodule number	1996	Israel	Burdman et al. 1996
	Bean	Ab Cd	Hydroponic	Increase lateral root Increase secretion of nod-gene inducing flavonoids	2008	Spain	Dardanelli et al. 2008
Bean	A.sp-co inoculated with <i>Bradyrhizobium</i>	Field			2013	Brazil	Hungria et al. 2013
Chickpea (<i>Cicer arietinum</i> L.) and Fava (<i>Vicia faba</i> L.)	Ab	Greenhouse and field	Enhanced nodulation by native rhizobia, improved root and shoot development		2001	Israel	Hamaoui et al. 2001
Chickpea	Al	Growth chamber	Improved growth, nutrient uptake and nodule formation		1989	Egypt	El-Mokadem et al. 1989
Soybean (<i>Glycine max</i> L.)	Ab	In vitro	Enhanced root function		1991	USA and Mexico	Bashan et al. 1990*, 1992*, Bashan 1991*
Soybean	Ab	Shadehouse	Increased nodulation and grain yield		1979	India	Singh and Subba 1979
Soybean	Ab	Growth chamber	Promoted seed germination, nodule formation, and early development of seedlings		2009	Argentina	Groppa et al. 1998; Cassan et al. 2009b
Soybean	A.sp	In vitro	Increased plant parameters and N ₂ fixation		1983; 2001	Belgium, Japan, Egypt	Inuthayathas et al. 1983; Galal 1997; Chebotar et al. 2001*
Soybean	Ab	In vitro	Increased plant parameters		2001	Malaysia	Molla et al. 2001a, b
Soybean	A.sp-co inoculated with <i>Bradyrhizobium</i>	Field			2013	Brazil	Hungria et al. 2013
Wild plants	Peanut (<i>Arachis hypogaea</i> L.)	Al	Field	Increased nodulation and N content	1988	India	Ravetkar and Konde 1988
	Oak (<i>Quercus ithaburensis</i> Decaisne)	Ab	Seedling development	1995	Israel	Zaady and Perevolotsky 1995
	Cacti <i>Pachyverus pringlei</i> (S. Watson) Britton & Rose, <i>Stenocercus thurberi</i> (Engelm.) Buxb. and <i>Lophocercus schottii</i> (Engelm.) Britton & Rose	Ab	Growth chamber, greenhouse, field	Enhanced germination, seedling development	1993–2012	Mexico	Puente and Bashan 1993*, Bashan et al. 1999*, 2009a,b, 2012
	<i>Salicornia bigelovii</i> Torr.	Al	Greenhouse	Enhanced growth parameters and seed quality	2000, 2003	Mexico	Bashan et al. 2000; Rueda-Puente et al. 2003
	Mezquite (<i>Prosopis juliflora</i> (SW.) DC.)	Ab CECT590	Nursery	Enhance plant growth	2012	Spain	Dominguez-Núñez et al. 2012
	<i>Casuarina equisetifolia</i> L.	Ab Cd	Field	Increase in height, stem, branch and root weight, total biomass. Increase in nutrient content	2004	India	Rajendran and Devaraj 2004

Table 1 (continued)

Type of plant	Plant species	bacterial species**	Test conditions	Effect on plant	Range of years of studies	country	References
	<i>Cistus albidus</i> L.	Ab mixed with <i>Pantoea dispersa</i>	Field	Increase in shoot and root dry weight, Increase in P and K content	2008	Spain	Schoebitz et al. 2014
Microalgae	<i>Chlorella vulgaris</i> Beijerinck, <i>Chlorella sorokiniana</i> Shihira et Krauss	Ab Cd, Ab Sp6, Ab Sp245.	In vitro	Increases population growth, pigments, lipids and carbohydrates concentration, enhances enzymatic activities	2002–2015	Mexico	de-Bashan et al., 2002, 2005*, 2008a*, b*, Choix et al., 2012a*, b*, 2014*, Leyva et al. 2014*, 2015*, Meza et al. 2015a*, b*.
	Plankton	A.sp	Fish farm	Increase in plankton population, increase in common carp growth	1999	India	Garg and Bhatnagar 1999

ND not detected

* Inoculated bacteria were re-isolated at the end of the experiment

** Ab=*Azospirillum brasilense*, Al=*A. lipoferum*, A.sp=*Azospirillum* sp., Ah=*A. halopraeferens*

to enhance growth and yield of vegetables, industrial crop plants (Bashan et al. 1989b, c; 1991), burr medic (*Medicago polymorpha* L.) seedlings (Yahalom et al. 1990), common bean (*Phaseolus vulgaris* L.) (Burdman et al. 1996), environmental plants (Bashan et al. 2009b, 2012) and sunflower (*Helianthus annuus* L.) (Itzigsohn et al. 1995). Additionally, *A. brasilense* Sp7 could attach to arbuscular mycorrhizal structures (Bianciotto et al. 2001). The most unexpected enhancement was that of the rootless, single cell microalgae *Chlorella vulgaris* Beijerinck and *C. sorokiniana* Shihira et Krauss; the resemblance to its effects on growth, photosynthesis and metabolite content of plants (Bashan and Dubrovsky 1996; Gonzalez and Bashan 2000; de-Bashan et al. 2002; Bashan et al. 2006; Choix et al. 2012a, b) and its phenotypic cell-cell attachment employing fibrills (de-Bashan et al. 2011) made the combination of this strain with microalgae a proposed general model for plant-bacterial interaction (de-Bashan and Bashan 2008).

Another strain interacting with multiple hosts and multiple activities on plants is *A. brasilense* Sp245, which is able to colonize the surface of roots (Pereg Gerk et al. 2000) despite the fact that it was originally isolated from surface sterilized wheat roots and was shown to colonize wheat roots as an endophyte (Baldani et al. 1983, 1986; Schloter et al. 1993; Assmus et al. 1995). The strain is able to increase nitrate assimilation (Ferreira et al. 1987), alter membrane potential and proton efflux (Bashan 1990; Bashan and Levanony 1991; Bashan et al. 1989a) and enhance nitrogen accumulation (Boddey et al. 1986) and yield of wheat (Baldani et al. 1987; Okon and Labandera-Gonzalez 1994). Subsequently, this strain was used for inoculation of other plants and was capable of enhancing their growth. Strain Sp245 could increase the hormone abscisic acid content in *Arabidopsis thaliana* (L.) Heynh plants (Dubrovsky et al. 1994; Cohen et al. 2008) and have even more attributes. Those include: increasing growth and mineral content in soybeans (*Glycine max* (L.) Merr.) (Bashan et al. 1990), improving vigor of aged seed of lettuce (*Lactuca sativa* L.) (Carrozzi et al. 2012) enhancing germination and growth of the giant cardon cactus (*Pachycereus pringlei* (S. Watson) Britton & Rose) (Puente and Bashan 1993), improving the establishment of three cactus species in the field (Bashan et al. 1999) and promoting the growth of the halophyte *Salicornia bigelovii* Torr (Bashan et al. 2000). It survived well in the rhizosphere of tomato (*Solanum*

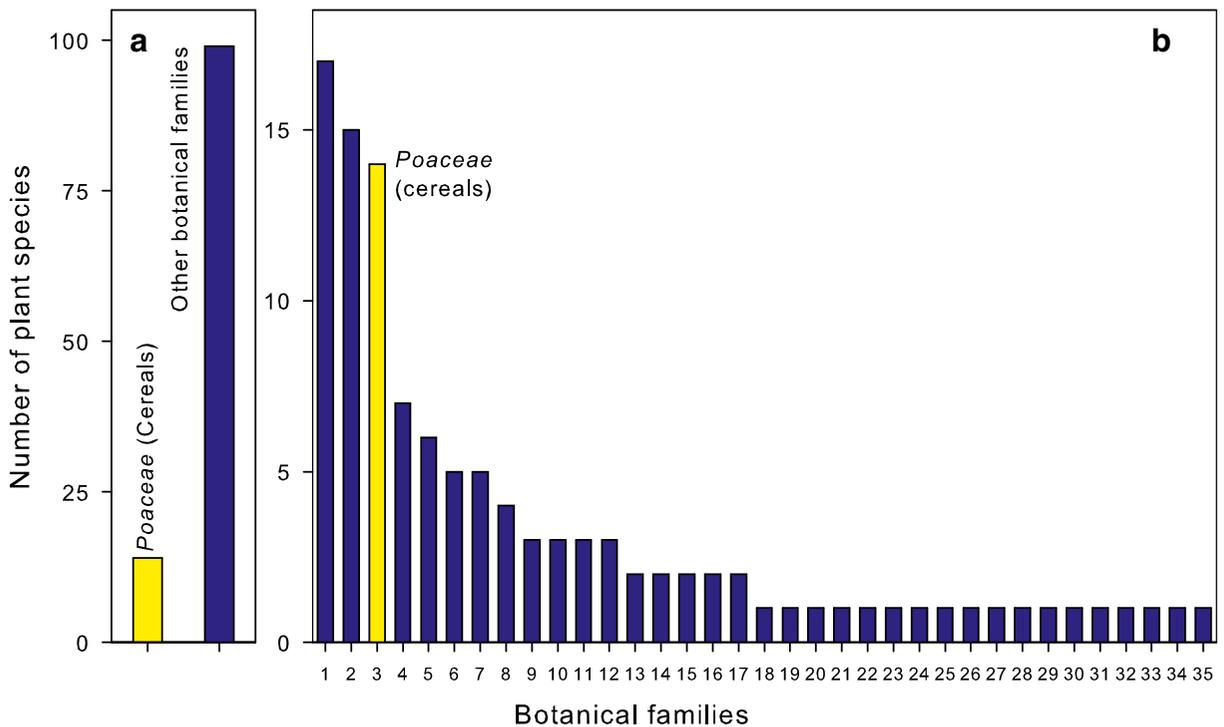


Fig. 1 Plant species, cereals vs. non-cereals inoculated with *Azospirillum* sp. showing plant beneficial effects (a). Botanical families on which *Azospirillum* sp. exerted beneficial effects (b). Families: 1. Asteraceae; 2. Fabaceae; 3. Poaceae (gramineas); 4. Brassicaceae; 5. Apiaceae; 6. Solanaceae; 7. Amaranthaceae; 8. Malvaceae; 9. Piperaceae; 10. Cucurbitaceae; 11. Rosaceae; 12. Cactaceae; 13. Euphorbiaceae; 14. Convolvulaceae; 15.

Caryophyllaceae; 16. Urticaceae; 17. Chlorophyceae; 18. Musaceae; 19. Casuarinaceae; 20. Cistaceae; 21. Linaceae; 22. Myrtaceae; 23. Phyllanthaceae; 24. Moraceae; 25. Fagaceae; 26. Arecaceae; 27. Pedaliaceae; 28. Acanthaceae; 29. Agavaceae; 30. Zingiberaceae; 31. Ranunculaceae; 32. Papaveraceae; 33. Iridaceae; 34. Geraniaceae; 35. Polygonaceae

lycopersicum L.) (Bashan et al. 1995), colonizing numerous weed species of different families (Bashan and Holguin 1995), inducing ammonium transporter in tomato root (Becker et al. 2002), mitigating salt effects on lettuce (Barassi et al. 2006) and promoted the growth of tomato, pepper (*Capsicum annuum* L.), and cotton (*Gossypium hirsutum* L.) (Bashan 1998; Bashan et al. 1989b, c; Bashan and de-Bashan 2005). Finally, it enhanced accumulation of intracellular nitrogen, phosphorus and enzymatic activities in a unicellular microalgae *Chlorella vulgaris* (de-Bashan et al. 2008b; Meza et al. 2015a, b).

A third example is *A. brasilense* Sp6. Originally isolated from maize, it promoted significant growth in maize (Barbieri and Galli 1993) and growth of roots of wheat (Barbieri and Galli 1993; Barbieri et al. 1986) and sorghum (*Sorghum bicolor* (L.) Moench) (Basaglia et al. 2003). The same isolate improved the growth of the shrub quailbush, *Atriplex lentiformis* (Torr.) S.Wats (de-Bashan et al. 2010a) and significantly changed the

metabolism of the microalgae *C. vulgaris* and *C. sorokiniana* (de-Bashan and Bashan 2008; Meza et al. 2015a, b).

A fourth example is *A. brasilense* Az39, the most common commercial strain in Argentina, was tested on several cereals with significantly improved yield results (Fulchieri and Frioni 1994; Cassan et al. 2009a; Díaz-Zorita and Fernández-Canigia 2009; Zawoznik et al. 2011; Garcia de Salamone et al. 2012; Masciarelli et al. 2013) or in combination with legume nodule microorganisms (Cervantes and Rodríguez-Barrueco 1991; Perrig et al. 2007). This strain was proven growth promoter for soybean (Cassan et al. 2009b), and *Casuarina* sp. (Rodríguez-Barrueco et al. 1991). Numerous local publications from Argentina and India indicate the successful use of this strain for sunflower, tomato, cucumber (*Cucumis sativus* L.), pepper, squash (*Cucurbita* sp. L.), cabbage (*Brassica oleracea* L.), radish (*Raphanus sativus* L.), cotton, peanuts (*Arachis hypogaea* L.), alfalfa (*Medicago sativa* L.), Achicoria

(*Cichorium intybus* L.), and the flowers of the day lily (*Hemerocallis lilioasphodelus* L.) and *Nierembergia linariaefolia* (Graham). (Table S1, supplementary material).

While there is evidence to show that strains of certain *A. brasilense* affect more than one group of plants, the main difficulties in assessing *Azospirillum* specificity or even affinity are the lack of studies methodically testing different strains of the same species (obtained from different sources) on specific host plant and specific strains on different host species.

Physiological and biochemical studies of attachment and initial colonization as parameters of potential affinity

Survival in the plant's rhizosphere and promotion of plant growth do not necessarily qualify the plant as a host. To be a true host, the plant has to harbor the bacteria attached to or inside its tissue, ensuring long-term association between the two. The ability of *Azospirillum* species to attach to plant roots in various ways is well documented (multiple reference in Table 2) making some plants genuine *Azospirillum* hosts.

Azospirillum-mediated plant growth promotion utilizes various mechanisms that clearly demonstrate benefits to the host plant from this association, of which four are particularly important. Firstly, if *Azospirillum* is not attached to root epidermal cells, growth promoting substances excreted by the bacteria diffuse into the rhizosphere, consumed by nutritionally versatile microorganisms before reaching the plant and there is no mutual beneficial interaction (Bashan 1986a). With physical attachment part of these substances is diffused into the intercellular spaces of the root cortex. Secondly, without a secure attachment, rain or irrigation water may dislocate the bacteria from the rhizoplane to perish in the surrounding, nutrient-deficient soil. *Azospirillum* poorly survives in many soils without host plants (Bashan et al. 1995; Bashan and Vazquez 2000). Thirdly, association sites on roots having no attached *Azospirillum* represent a target for other aggressive rhizosphere colonizers, which are not necessarily beneficial for the plant. Finally, the root provides *Azospirillum* with microaerophilic niches that suit the physiological properties of this genus. *Azospirillum* proliferates under both aerobic and anaerobic conditions, but is preferentially microaerophilic in the presence or absence of combined

nitrogen in the medium (Okon and Itzigsohn 1992) and it shows a strong aerotactic response towards the zones with reduced oxygen tension on roots (Okon et al. 1980; Patriquin et al. 1983; Reiner and Okon 1986; Zhulin et al. 1996; Alexandre et al. 2000; Stephens et al. 2006). There does not appear to be any evidence to suggest that *Azospirillum* is harmful to plants (Bashan 1998). The association between *Azospirillum* and the plant can thus be defined, in general, as mutualism suggesting a possible host specificity involved in this mutualistic relationship between the attached *Azospirillum* cells and the host at any level (genus, species or strain). The mode of attachment of *Azospirillum* to its host plant, as well as to other substrates, is an essential element to be ascertained.

Azospirillum strains can colonize roots externally and/or internally or can colonize the stem as an endophyte, as seen in rice (*Oryza sativa*), while some strains doing both (Table 2; Ramos et al. 2002; Zhu et al. 2002; Xie and Yokota 2005). Fluorescently labeled probes and monoclonal antibodies have confirmed the presence of *Azospirillum* strains in both the plant interior and the rhizosphere (Schloter et al. 1993; Assmus et al. 1995). Specifically, *A. brasilense* strain Sp245 was found in the root xylem, while Sp7 could only be detected on the root surface (Schloter et al. 1994). X-gal staining of labeled bacteria revealed that strain Sp7 initially colonized the sites of lateral root emergence and the root hair zone (Katupitiya et al. 1995a; Pereg Gerk et al. 2000) as does strain Sp245 (Vande Broek et al. 1993; Pereg Gerk et al. 2000). Washing wheat roots colonized by *A. brasilense* Cd removed most of the root-external bacteria and revealed a smaller internal root population (Bashan et al. 1986). *A. brasilense* Cd was also detected internally, within the cortex, using immuno-gold labeling (Levanony et al. 1989).

Root surface colonization is more common, in which the bacteria form small aggregates, although many single cells are scattered on the root surface. These surface colonizers are embedded in the external mucigel layer of the root (Umali-Garcia et al. 1981; Berg et al. 1979; Schank et al. 1979; Bashan et al. 1986; Murty and Ladha 1987; Pereg Gerk et al. 2000). Interestingly, both live and dead roots can be colonized (Bashan et al. 1986) suggesting that, while *Azospirillum* is attracted to root exudates, bacterial-host signaling is not essential for the actual attachment to roots, making a saprophytic growth phase of this bacterium probable.

When examining colonization by various strains, factors other than specificity can influence colonization

and therefore can be responsible for differences among strains in separate studies. Factors important for such consideration include the culture age, experimental procedures and/or environmental conditions (multiple references in Table 2), and even the presence of other endophytic bacteria on the roots (Bacilio-Jimenez et al. 2001). In addition, some strains isolated from plant or rhizosphere (thus considered as environmental strains), such as *A. brasilense* strains Sp6 and Sp35 and *A. lipoferum* strains RG20, S28, and Br17 were not able to aggregate/flocculate (Pereg Gerk et al. 1998). Flocculation is both related to the production of exopolysaccharide (EPS) in *Azospirillum* and considered to be essential for firm attachment to root surface, suggesting that effects on plants observed following inoculation with these strains were not necessarily due to attachment to the roots but rather to the presence of these strains in the rhizosphere.

Azospirillum preferentially colonizes root elongation zones, root-hair zones and emergence of lateral roots with colonization patterns that depend on the host plant and bacteria strain (Bashan et al. 1986; Okon and Kapulnik 1986; Assmus et al. 1995; Pereg Gerk et al. 2000; Trejo et al. 2012). In wheat, colonization is mainly on the root surface and very few bacteria are attached to the root hairs (Okon and Kapulnik 1986; Bashan and Levanony 1989b), whereas in rice, massive root-hair colonization was frequently observed (Murty and Ladha 1987). In pearl millet (*Pennisetum glaucum* (L.) R.Br.) (Matthews et al. 1983), Kallar grass (*Panicum antidotale* Retz.) (Reinhold et al. 1986) and sugarcane (*Saccharum* sp.) callus (Berg et al. 1979; Vasil et al. 1979) most of the *Azospirillum* population was concentrated on the root surface. The colonization sites in some grasses corresponded to the areas where root mucigel was present, while the area around the point of emergence of lateral roots usually shows high colonization (Bilal et al. 1993).

It is clear that various plants can host bacteria from the genus *Azospirillum* and, evidence shows that species and even strains of *Azospirillum* can colonize more than one plant, suggesting a wide host range for each species/strain. Despite this, the colonization of plants has been visually demonstrated with only few strains of *Azospirillum* (Table 2) and a more thorough investigation of a large number of strains is required in order to conclude regarding host specificity/affinity of *Azospirillum* strains.

Mechanisms of attachment in relation to potential affinity

Examination of the mechanisms of attachment of *Azospirillum* to various substrates compared with plant roots better addresses the specificity of the interaction with hosts. Electron microscopic studies on several plant species have demonstrated that *Azospirillum* cells are connected to the root surface and to each other within the bacterial aggregate by a massive network of fibrillar material (Bashan et al. 1986; Levanony et al. 1989). Although azospirilla do not always show a uniform pattern of attachment in different experiments, even when the same strain is used on the same host (Michiels et al. 1989), it seems that aggregation by fibrillar material is the characteristic root surface colonization of this genus regardless the species or the strain (Umali-Garcia et al. 1980; Patriquin et al. 1983; Bashan et al. 1986; Gafni et al. 1986; Okon and Kapulnik 1986; Hadas and Okon 1987; Levanony et al. 1989). The chemical nature of these fibrils is not fully defined, but there are indications that they contain proteinaceous compounds (Bashan and Levanony 1989b) and polysaccharides that are responsible for the attachment phenomenon (Katupitiya et al. 1995a; Pereg Gerk et al. 1998, 2000). Fibrillar attachment by the bacteria is primarily dependent on active bacterial metabolism; killed bacteria did not attach to roots, but live bacteria attached to dead plant material (Bashan et al. 1986; Gafni et al. 1986). Initial root surface attachment is relatively weak and a slight rinsing of the roots releases most of the bacteria (Bashan et al. 1986) probably because of cell surface hydrophobicity, cell surface charges and cell surface lectins (Castellanos et al. 1997, 1998). Less thermodynamically stable polar attachment of *Azospirillum* cells to roots (Patriquin et al. 1983; Whallon et al. 1985; Levanony et al. 1989) comprised only a small fraction of the cells. Most of the root surface was colonized by bacteria in a horizontal, thermodynamically more stable, position. Examination of several strains of *A. brasilense* and *A. lipoferum* showed that although surface colonizers and endophyte strains had similar ability to anchor to wheat roots, strains with a proven ability to invade the root interior were more competitive in attaching to adsorption sites (de Oliveira Pinheiro et al. 2002).

Azospirillum has two different phases of attachment to wheat roots. The primary adsorption phase is fast but weak, reaching a maximum within 2 h of incubation,

Table 2 Studies that showed actual attachment of *Azospirillum* strains to host roots, host cells or inert surfaces

<i>Azospirillum</i> strain	Host plant	Reference
<i>A. brasilense</i>		
Sp13t, Sp7, JM125A2	Guinea grass (<i>Panicum máximum</i> Jacq.), pearl millet (<i>Pennisetum glaucum</i> (L.) R.Br.)	Umali-Garcia et al. 1980
Sp7 and sp245, Sp107, JM6A2, JM73B3, SpBr14	Wheat (<i>Triticum aestivum</i> L.)	Jain and Patriquin 1984
Cd, Cd-1	Different cultivar wheat	Kapulnik et al. 1983
245, Sp7, 7030	Isolated plant cells of <i>Zinnia elegans</i> Jacq. and <i>Triticum monococcum</i> L.	Eyers et al. 1988a
Cd and SpBr14, Sp7 Sp59b	Rice (<i>Oryza sativa</i> L.) and clover (<i>Trifolium</i> sp. L.)	Sukiman and New 1990
Sp245 [gusA]	Wheat	Vande Broek et al. 1993
JM82	Kallar grass (<i>Leptochloa fusca</i> (L.) Kunth) and rice	Bilal et al. 1993
Sp7, Cd, Sp13, SpBr14, Sp245, Sp107, 576	Wheat + -2,4,D	Katupitiya et al. 1995b
245, Sp7, Cd	Wheat	Zamudio and Bastarrachea 1994
Sp245, Sp7, Wa3	Wheat	Assmus et al. 1995
Sp7	Wheat	Vande Broek et al. 1998
Sp245 internal, Sp7 and Wa5 surface	Two wheat cultivars (Differences between cultivars)	Schloter and Hartmann 1998
Sp245, 75 and 80 from Russia	Different wheat cultivars (Differences with bacterial growth phase and cultivar)	Yegorenkova et al. 2001
Sp7	Rice roots	Zhu et al. 2002
Sp245, Sp7	Various wheat cultivars	Rothballer et al. 2003
REC3, PEC5	Strawberries (<i>Fragaria ananassa</i> (Weston) Duchesne)	Guerrero-Molina et al. 2012
SM	<i>Sorghum bicolor</i> (L.) Moerch	Kochar and Srivastava 2012
AbV5, AbV6	Corn (<i>Zea mays</i> L.) Protected from insect herbivore <i>Diabrotica speciosa</i> Germar	Santos et al. 2014
Sp7, Cd, Sp245, Sp246, Sp107, SpBr14, JA2	Wheat	de Oliveira Pinheiro et al. 2002
<i>A. brasilense</i> Cd and <i>A. halopraeferens</i> AU10	Blank mangrove (<i>Avicennia germinans</i> L.)	Puente et al. 1999
Cd	Wheat, sorghum	Bashan et al. 1986; Bashan and Levanony 1989a,b; Levanony et al. 1989; Trejo et al. 2012
Cd	Tomato (<i>Solanum lycopersicum</i> L.), eggplant (<i>Solanum melongena</i> L.), pepper (<i>Capsicum annum</i> L.), cotton (<i>Gossypium hirsutum</i> L.), soybean (<i>Glycine max</i> L.)	Bashan et al. 1989c, 1991; Levanony and Bashan 1991.
Cd	Soil, sand and peat particles, polystyrene, alginate beads; several rock particles; glass	Bashan 1986b; Bashan and Levanony 1988a, b; Bashan et al. 1991; Bashan and Holguin 1993; de-Bashan et al. 2002; Wisniewski-Dye et al. 2011
Cd	Microalgae (<i>Chlorella vulgaris</i> Beijerinck, <i>C. sorokiniana</i> Shihira et Krauss)	Lebsky et al. 2001; de-Bashan and Bashan 2008; de-Bashan et al. 2011
Sp6	Quailbush (<i>Atriplex lentiformis</i> (Torr.) S. Watson)	de-Bashan et al. 2010a, b
<i>A. lipoferum</i>		
Sp59b	Wheat	Jain and Patriquin 1984
4B	Rice roots	Chamam et al. 2013
34H	Rice roots	Murty and Ladha 1987
SpBr17, 596, Sp59b	Wheat + -2,4,D	Katupitiya et al. 1995b

Table 2 (continued)

<i>Azospirillum</i> strain	Host plant	Reference
SpBr17	Wheat	Zamudio and Bastarrachea 1994
S82, SpBr17, Sp59	Wheat	de Oliveira Pinheiro et al. 2002
<i>A. irakense</i>		
KBC1 Shown as a vibriod – is it a wild type?	Rice roots	Zhu et al. 2002
<i>Azospirillum</i> sp.		
<i>Azospirillum</i> sp B510	Rice Suppressed fungal and bacterial pathogens	Yasuda et al. 2009

and likely governed by bacterial proteins. The second or anchoring phase takes longer, beginning after 8 h of incubation and reaching a maximum after 16 h, is stronger and irreversible, and appears to involve bacterial extracellular surface polysaccharides yielding long fibrils and a large amount of mucigel-like substances (Umali-Garcia et al. 1980; Zaady and Okon 1990; Gafni et al. 1986; Bashan and Levanony 1988b; Eysers et al. 1988a, b; Del Gallo and Haegi 1990; Michiels et al. 1990; 1991; Bashan et al. 1991; Levanony and Bashan 1991; Skvortsov et al. 1995; Puente et al. 1999). This type of attachment is not only to roots but also to plants cells as in the case of production of anchoring material when *A. brasilense* interacts with the single cell aquatic microalgae *Chlorella vulgaris* (de-Bashan et al. 2011). During this phase movement of *Azospirillum* along the root surface is minimal owing to formation of multi-stranded fibrils, although several single cells are capable of migrating among the different sections of the root system (Bashan 1986b; Bashan and Levanony 1991; Bashan and Holguin 1994) and among individual plants (Bashan and Levanony 1987; Bashan and Holguin 1995). These holdfast fibrils ensure vertical bacterial transfer by the growing root tip to deeper soil layers (Bashan and Levanony 1989a, 1991).

Adsorption and anchoring are probably different phenomena (Michiels et al. 1990, 1991) and have been observed in the roots of tomato, pepper, cotton, and soybean (Bashan et al. 1989b, c, 1991). The polar flagellum of *A. brasilense*, which is primarily used for swimming, was also involved in the initial attachment process of the bacteria to roots (Croes et al. 1993).

Several physiological, environmental, nutritional, and chemical factors modify *A. brasilense* attachment

to the roots. Lectin and hydrophobic binding have been suggested as possible mechanistic mediators (Umali-Garcia et al. 1980; Tabary et al. 1984; Antonyuk et al. 1993; Karpati et al. 1995; Castellanos et al. 1997, 1998, 2000).

There are at least two different quantitative types of anchoring by this bacterium: a weak attachment to a non-biological surface and a stronger attachment to roots even though microscopically they resemble each other. The anchoring of *A. brasilense* Cd to hydrophobic polystyrene was significantly less than to roots and this is likely due to the hydrophobicity of the polystyrene (Bashan and Holguin 1993). Although most inoculated *Azospirillum* spp. survived only for a limited time in the soil (Bashan et al. 1995), some strains are soil dwellers especially in the tropics (Döbereiner et al. 1976; Döbereiner 1988). Upon inoculation to the soil *Azospirillum* cells are usually irreversibly adsorbed by the upper fraction of the soil profile in a charge-charge interaction mainly with clays and organic matter. Later they form attachments to soil particles such as sand, organic matter and clays using fibrillar material in a manner similar to attachment to roots. Physical and chemical soil conditions such as pH, flooding, dry regime, and availability of bacterial chemo-attractants greatly affect adsorption of *Azospirillum* to different degrees (Bashan and Levanony 1988a; Horemans et al. 1988). Attachment of *Azospirillum* to pure sand, which lacked clays and organic matter, was weaker and accomplished by a network of protein bridges produced between the bacteria cell and the quartz particles and mainly controlled by nutrient availability (Bashan and Levanony 1988b). Out of several strains examined, attachment to glass mediated by pili seems to be

exclusive to *A. brasilense* Sp245 (Wisniewski-Dyé et al. 2011).

In summary, the phenotypic attachment of *Azospirillum* is mainly characterized by two-phase attachment, perhaps with the aid of the polar flagellum and a higher affinity of the attachment process to roots rather than to inert or soil surfaces. However, evidence suggests that there is a close resemblance between *Azospirillum* attachments to the root surfaces of different plant species, to the surfaces of single cells and to soil particles. Consequently, attachment to roots is potentially unsuitable for assessing the specificity at the species or strain levels. Affinity to roots in general may be concluded but there is insufficient biochemical and physiological data to suggest a particular affinity to cereal roots.

As a final note, endophytic *Azospirillum* strains may be the key to understanding the specificity of these strains to particular hosts, since here the bacterium has progressed beyond the attachment stage to enter the root system (for example, *A. brasilense* Sp245 in wheat roots, (Schloter et al. 1994) or the stem (for example *A. oryzae* COC8 and its relative *Azospirillum* sp. B510, Kaneko et al. 2010). Such advanced interaction suggests that the endophytic strains may communicate with the plant during the process of infection. Unfortunately, despite observations of internal colonization and advances in genomics, there is no information available on the actual mechanism of internal colonization by *Azospirillum* and its regulation. Further examination of *Azospirillum* at the molecular level may provide insights despite this lack of mechanistic information.

Molecular *Azospirillum*-plant interaction as potential indicator of affinity of *Azospirillum* strains

To date, only a small cohort of genes and molecular factors has been investigated for their involvement in the interactions between *Azospirillum* and plants, particularly those pertaining to potential insights regarding specificity/affinity. The lack of an easily detected plant phenotype that could be used to select bacterial mutants after inoculation with *Azospirillum* has complicated investigation of the genetic basis for the interaction. Consequently, mutations in traits considered to play a role in plant association have been mostly investigated and include genes involved in the production of auxins

and surface compounds, genes sharing DNA homology with other plant-associative bacteria, such as *nod*, involved in nodulation by *Rhizobium* (Onyeocha et al. 1990), *Rhizobium* *exo* genes, involved in EPS production (Michiels et al. 1988; Petersen et al. 1992) and genes responsible for nitrogen fixation (*nif* and *fix* genes) (Vande Broek and Vanderleyden 1995). The p90 megaplasmid of *Azospirillum* was shown to carry genes such as *exoBC*, *nodPQ*, *mot1,2,3* genes (production of polar and lateral flagella) and genes involved in IAA synthesis and in chemotaxis (Michiels et al. 1989; Katsy et al. 1990; Onyeocha et al. 1990; Van Rhijn et al. 1990; Vieille and Elmerich 1990; Elmerich et al. 1991; De Troch et al. 1994). Although no role was found for the *nodPQ* and *exoBC* homologous genes in *Azospirillum* colonization of roots, the plasmid p90 was named the rhizocoenotic plasmid, pRhico by Croes et al. (1991).

Attachment of *Azospirillum* to wheat roots is mainly dependent on two factors: the existence of a polar flagellum that allows the bacteria to attach to the roots and produce EPS, allowing bacteria to firmly attach to the root surface (Michiels et al. 1990, 1991; Croes et al. 1993). EPS production is regulated by the *flcA* gene, although the mechanism by which this regulation occurs is not fully understood (Pereg Gerk et al. 1998). This is the only regulatory gene that is known to be related to the attachment process.

The response regulator protein, FlcA, controls the shift of *Azospirillum* from vegetative state to cyst-like forms, both in cultures and in association with plants. Tn5 transposon-induced *flcA*⁻ mutants do not flocculate, do not transform from motile vibrioid cells into non-motile cyst-like forms and lack the EPS material on the cell surface under all conditions (Pereg Gerk et al. 1998). This leads to significantly reduced colonization efficiency of plant roots by *Azospirillum*, as they depend on the production of EPS to firmly attach to the root surface (Katupitiya et al. 1995b; Pereg Gerk et al. 1998, 2000). Development of reliable RT-PCR reference genes for *Azospirillum* (McMillan and Pereg 2014), facilitated the demonstration that *flcA* is involved in both the stress response and carbohydrate and nitrogen metabolism in *Azospirillum* during flocculation (Hou et al. 2014). Proteomics, RT-PCR (Hou et al. 2014) and cDNA-AFLP (Valverde et al. 2006) analyses to compare wild type *A. brasilense* Sp7 and non-flocculating *flcA*⁻ mutants, have identified genes and proteins involved in the flocculation and aggregation of strain

Sp7 (Hou et al. 2014), amongst which was a chemotaxis-like *che1* homologue. Interestingly the Che1 pathway has been suggested to play a role in the adhesive cell properties of *A. brasilense* (Siuti et al. 2011) and controls swimming velocity, which affects transient cell-to-cell clumping (Bible et al. 2012). Similarly the nitrite/nitrate transporter NarK appears involved in aggregation (Valverde et al. 2006; Hou et al. 2014) and interestingly, the *narL* homologue, which possibly regulates respiratory membrane-bound nitrate-reductase, is highly expressed in wheat-bound *A. brasilense* FP2 cells (Camilios-Neto et al. 2014). While it has been established that *flcA* responds to environmental cues (Pereg Gerk 2004), its expression in response to various hosts has yet to be established. Further analysis of the conditions that affect *flcA* expression may shed light on *Azospirillum* affinity for various hosts.

Surface properties of *Azospirillum* are important in the attachment process. Two genes, *noeJ* (mannose-6-phosphate isomerase) and *noeL* (GDP-mannose 4,6-dehydratase), were related to EPS biosynthesis and biofilm formation (Lerner et al. 2009), and are present in the genome of several strains of *Azospirillum* (Sant'Anna et al. 2011; Wisniewski-Dyé et al. 2012). Disruption of dTDP-rhamnose biosynthesis by Tn5 mutagenesis modified the lipopolysaccharide core, EPS production and root colonisation in *A. brasilense* Cd (Jofre et al. 2004). Tlp1, an energy transfer transducer is required for taxis and for colonisation of wheat roots by *A. brasilense* Sp7 (Greer-Phillips et al. 2004). The pili mutant (*cpaB*⁻) of *A. brasilense* Sp245 has reduced biofilm formation and attachment to glass (Wisniewski-Dye et al. 2011). All of the above may be important in attachment to host roots. It is interesting to note that a spontaneous mutant of *A. brasilense* Sp7 lacking its lateral flagella, namely PM23, lost its ability to swarm through semi-solid medium but was able to attach to root surface (Pereg Gerk et al. 2000). Comparative analysis of the genomes of various species and strains of *Azospirillum* may link the molecular motifs related to attachment with the ability of different species to colonise different hosts as well as explain the different modes of colonisation discussed above (root surface versus root interior colonization).

Despite the great interest in this bacterium and its use as commercial inoculant (Bashan et al. 2014; Calvo et al. 2014), investigations into the molecular traits involved in its attachment to plants have been lacking.

The value of such research was identified almost three decades ago (Elmerich et al. 1987) but the paucity of such studies persists to the present.

New insight into *Azospirillum*–plant interactions using high throughput technology: genomics and transcriptomics in relation to affinity for plants

The *Azospirillum* strain B510, an isolate of rice and a close relative of *A. oryzae*, was the first complete genomic analysis of an *Azospirillum* strain published (Kaneko et al. 2010). Subsequently, the growing number of genomic sequences available for *Azospirillum* species has facilitated valuable comparative studies, potentially illuminating the specificity of the *Azospirillum*-host interaction.

The detection of a tripartite ATP-independent periplasmic transport system and a diverse range of malic enzymes in the genome of *Azospirillum* strain B510 were implicated in the utilisation of C4-dicarboxylate during its interaction with rice (Kaneko et al. 2010). Genomic analysis of *A. brasilense* Sp245 suggested transition of the genus from aquatic to terrestrial environments at approximately the same time as the emergence of vascular plants on land (Wisniewski-Dye et al. 2011). A proposed high frequency of horizontal gene transfer (HGT) from soil and plant-associated bacteria is the suggested mechanism of adaptation to the rhizosphere and to the host plant. Such a mechanism would allow the bacteria to gain, rearrange and lose genetic traits as required for success in their ecological niche. Indeed, a high frequency of plasmid DNA rearrangements was reported for *A. brasilense* Sp7 that affected biofilm formation on glass and roots (Petrova et al. 2010). Moreover, higher genomic plasticity was shown in *Azospirillum* genomes compared to rhizobial genomes known for their genome plasticity (Wisniewski-Dye et al. 2011), strengthening the suggested link between the appearance of phenotypic variants and plasmid loss or reorganization (Vial et al. 2006b). High genomic plasticity supports the suggestion that *Azospirillum* possesses mechanisms of adaptation to its various hosts.

The demonstration that the *Azospirillum* genome acquired a substantial number of glycosyl hydrolases by HGT that are essential for decomposition of plant cell walls and that the *A. brasilense* Sp245 genome contains three enzymes that are orthologous to cellulases

(Wisniewski-Dyé et al. 2011), supports previous suggestions that cellulolytic activity may be crucial to the ability of some *Azospirillum* strains to penetrate plant roots (Skvortsov and Ignatov 1998). A comparison between whole genomes of four *Azospirillum* strains, *A. brasilense* strains CBG497 (from maize grown on alkaline soil) and Sp245, *A. lipoferum* 4B and *Azospirillum* sp. B510 (Wisniewski-Dyé et al. 2012), reveal that the *Azospirillum* core genome (AZO-core) is dominated by proteins of ancestral origin (74 %) and 22 % of proteins acquired by HGT. While the 62–65 % of the AZO-core (strain dependent) is mainly chromosomally-encoded, the non-chromosomal proportion of AZO-core is unevenly distributed among strains. Several strain-specific genes were found to be involved in the colonization of plant roots; in a comparison among the four strains, additional flagellation and chemotaxis operons were found in *A. lipoferum* 4B and in *Azospirillum* sp. B510, while additional genes involved in EPS biosynthesis and/or transport and LPS biosynthesis were present in the *A. brasilense* strains, possibly acquired by HGT (Wisniewski-Dyé et al. 2012). Such strain-specific genes may suggest variability in the mode of interaction of strains with their hosts. It remains unclear, however, what effect, if any, this has on host specificity.

Although only four *A. lipoferum* strains out of 40 *Azospirillum* strains demonstrated acyl-homoserine lactone (AHL) biosynthesis ability (Vial et al. 2006a), it was suggested that strain-specific quorum sensing regulates functions linked to rhizosphere competence and adaptation to plant roots in *Azospirillum* (Boyer et al. 2008). Indeed, the genome analysis of *A. amazonense* Y2 revealed the presence of genes encoding for LuxI and LuxR homologs proteins suggesting it could synthesize AHLs and respond to their presence in the environment. The genome of *A. amazonense* also presents a *Klebsiella pneumoniae* *ahlK* homologue, possibly encoding a putative homoserine lactonase implicated in AHL degradation (Sant'Anna et al. 2011).

Although no difference was found in the anchoring ability of surface colonizers and endophytes of *A. brasilense* and *A. lipoferum* (de Oliveira Pinheiro et al. 2002), genomic comparisons suggest that *Azospirillum* strains have gained different root-adhesion mechanisms (Wisniewski-Dyé et al. 2012). Tight adherence (TAD) pili, essential for colonization and biofilm formation, are exclusive to the *A. brasilense* species while genes involved in cellulose synthesis

probably acquired by HGT are found exclusively in *A. lipoferum* 4B and *Azospirillum* sp. B510. Specificity was also found in the range of cellulases and hemicellulases produced by the different strains, with both *A. brasilense* strains encoding glycosyl hydrolase-encoding genes with no orthologues in any other *Azospirillum* genomes. *A. lipoferum* 4B and *Azospirillum* sp. B510 seem to be more versatile for aromatic compound degradation than *A. brasilense* strains, with a wider range of aromatic ring-hydroxylating dioxygenases, proposed to be related to the composition of the host plant exudates, as a result of niche-specific adaptation and environmental conditions (Wisniewski-Dyé et al. 2012).

Profiling of plant secondary metabolites of maize–*Azospirillum* (Walker et al. 2011) and rice–*Azospirillum* (Chamam et al. 2013) associations revealed strain-specific responses and suggest specific interaction between *Azospirillum* strains and their original host cultivar. The response of two *Oryza sativa japonica* (rice) cultivars, Cigalon and Nipponbare, to a root surface colonizer *A. lipoferum* 4B (isolated from Cigalon) and an edaphytic *Azospirillum* sp. B510 (isolated from Nipponbare), investigated using root transcriptome profiling, revealed not only strain-specific responses of rice, but also combination specific responses (Droque et al. 2014). Most of the differentially expressed genes were related to primary metabolism, transport and gene regulation; however, strain specific response was also observed for genes related to auxin and ethylene signaling suggesting complex response to hormone signaling (Droque et al. 2014). When considering differentially expressed genes to that of un-inoculated plants, inoculations by *Azospirillum* lead to the expression of genes related to stress response and plant defense in both rice inoculated with *A. lipoferum* 4B (Droque et al. 2014) and wheat inoculated with *A. brasilense* FP2 (Camilios-Neto et al. 2014). However, inoculation of rice by the endophytic strain B510 seems to lead to the repression of a wider set of genes than *A. lipoferum* 4B (Droque et al. 2014). A possible explanation is the ability of this strain to colonize the plant internally. Specificity may occur in the molecular responses of *Azospirillum* strains to their hosts, even though *Azospirillum*, as a genus, can interact with a wide range of hosts. Further investigation is required to explain the cellular changes of specific strains during association of this PGPB with plants.

Conclusions

Analysis of the extensive published data over four decades has facilitated certain conclusions about the specificity and/or affinity of organisms in the genus *Azospirillum*. This genus represents general plant growth-promoting bacteria improving the growth of 113 plant species of 35 botanical families, without any solid evidence of species specificity to selected plant species. Previous claims for specificity/affinity of *Azospirillum* for cereals, proposed in the early days of the field, are unsupported and may represent historical assumptions, likely related to the original isolation of *Azospirillum* organisms from cereals and experimentation performed almost exclusively on cereals. Numerous studies on *Azospirillum*-cereals association merely reflect the economic importance of cereals as crops, resulting in inaccurate claims that the main effect of this genus is on cereals. The demonstration of *Azospirillum*-plant interactions that stretch beyond cereals is expected to drive future research and greatly expand our knowledge of this important crop-enhancing genus.

It remains to be seen; however, the degree to which affinity may exist between different *Azospirillum* strains and various plant species, as does exist for many other PGPB for which specific strains performed better with specific plant genotypes or cultivars. Regardless of the wealth of research on *Azospirillum*-plant interactions, there is insufficient data regarding comparative bacterial strains-plant species or bacterial strain-plant genotypes. Additionally, comparative molecular analysis of different strains is in its infancy because only a handful of strains of *Azospirillum* are fully sequenced.

Future potential useful lines of research in this topic

Although no new specificity of *Azospirillum* to specific plant species is expected to emerge, the affinity of strains to plant genotypes or to plant species is worth investigating. This will involve examination of:

- A large number of strains tested on one plant species or a specific strain tested on multiple hosts and isolation of the strain after plant growth are required to propose affinity of *Azospirillum* strains.
- Molecular comparison at the entire genome level should be done between strains claimed to have affinity to specific plant species or to plant genotypes.

- A comparison regarding the differences between flocculating wild-type strains and non-flocculating mutants (often impaired in colonization) of the same strain regarding their effects on plant.
- A comparison between motile strains and strains naturally impaired in motility (affected by production of polar flagella that are required for initial *Azospirillum*-root interaction) on plants exuding large amount of exudates and those limited in the quantity and variability of these exudates.
- A comparison of cross inoculation between strains of different geographical origin (tropical, desert, temperate, aquatic) and plants from the same zone or with plants with *Azospirillum* strains isolated from another origin.

So far, the lack of knowledge regarding the specificity/affinity of *Azospirillum* does not detract from the numerous companies that offer commercial products for inoculation with *Azospirillum*. This knowledge will make possible tailoring of a better, future market product for sustainable agriculture in common agriculture practices and organic farming.

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