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Review Article

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Unraveling *Azospirillum*'s colonization ability through microbiological and molecular evidence

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Abstract

It is known that members of the bacterial genus *Azospirillum* can promote the growth of a great variety of plants, an ability harnessed by the industry to create bioproducts aimed to enhance the yield of economically relevant crops. Its versatile metabolism allows this bacterium to adapt to numerous environments, from optimal to extreme or highly polluted. The fact of having been isolated from soil and rhizosphere samples collected worldwide and many other habitats proves its remarkable ubiquity. *Azospirillum* rhizospheric and endophytic lifestyles are governed by several mechanisms, leading to efficient niche colonization. These mechanisms include cell aggregation and biofilm formation, motility, chemotaxis, phytohormone and other signaling molecules production, and cell-to-cell communication, in turn, involved in regulating *Azospirillum* interactions with the surrounding microbial community. Despite being infrequently mentioned in metagenomics studies after its introduction as an inoculant, an increasing number of studies detected *Azospirillum* through molecular tools (mostly 16S rRNA sequencing) as part of diverse, even unexpected, microbiomes. This review focuses on *Azospirillum* traceability and the performance of the available methods, both classical and molecular. An overview of *Azospirillum* occurrence in diverse microbiomes and the less-known features explaining its notorious ability to colonize niches and prevail in multiple environments is provided.

Keywords: plant growth promoting rhizobacteria, colonization, rhizosphere, microbiome, *azospirillum*

Introduction

Azospirillum is an α-proteobacterium belonging to the *Rhodospirillales* order and *Azospirillaceae* family (Baldani et al. 2015). Though its first description under the name of *Spirillum* dates from nearly a century (Beijerinck 1925), this microbe began to play a leading role in the agriculture scenario after the pioneer reports of Von Bulow and Döbereiner (1975) and Döbereiner et al. (1976), who first documented N_2 fixation in the roots of field-grown maize and the isolation of N_2-fixing *Spirillum lipoferum* Beijerinck from grass and cereal roots, as well as soil samples collected in several African countries and Brazil. A few years later, *Azospirillum* was proposed as a genus, and two species were described: *Azospirillum lipoferum* and *Azospirillum brasilense* (Tarrand et al. 1978). Since then, many bacteria within this genus have been identified and reclassified. To date, the genus consists of 27 confirmed species. Recently, two of the most studied strains were reclassified as new species based on genome analyses: *A. brasilense* Sp245 and other strains are now members of the new species *Azospirillum baldaniorum* (dos Santos Ferreira et al. 2020), and *A. brasilense* Az39 and similar strains are now part of the species *Azospirillum argentinense* (dos Santos Ferreira et al. 2022).

To date, 110 genomic sequences have been annotated for *Azospirillum* in the NCBI database (https://www.ncbi.nlm.nih.gov/data-hub/genome/?taxon=191). Even when not all these sequences are complete, some general features can be mentioned based on the available information. Members of the genus may have up to 10 megareplicons, including the chromosome, plasmids, and chromids, and these elements can be up to 1.12 megabases long. *Azospirillum* genomes usually comprise 6.33 and 8.1 megabases, with G-C contents varying between 68.2% and 70.7%. They have multiple copies of the 16S rRNA gene, which are heterogeneous within a single genome (Maroniche et al. 2016). More than 7 000 genes, 7 000 proteins, and 300 pseudogenes have been identified in this genus. *Azospirillum* contains a core genome that codifies 2 328 proteins, representing 30%–38% of total proteins. These core proteins have mostly (74%) an ancestral origin (Wisniewski-Dyé et al. 2012). The non-ancestral part of core proteins is codified by genes involved in signal transduction, carbohydrate and amino acid metabolism, and transport and adaptability to changing environments (Wisniewski-Dyé et al. 2015), like the soil and the rhizosphere.

Azospirillum spp. comprise Gram-negative, aerobic bacilli, motile, and chemotrophs. These versatile chemotrophs can use different substrates as carbon sources and grow in the presence of 3% NaCl; the optimum growth temperature varies between 33 and 41°C. The members of this genus produce catalase and oxidase, reduce nitrates into nitrites, degrade urea into ammonia, produce indoles, modify soil urease activity, and alkalinize milk. They can also reduce acetylene and test

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positive for arabinose and fructose fermentation (Baldani et al. 2015).

Azospirillum has been widely known for its plant growth promotion abilities ascribed to mechanisms such as N biological fixation and production of phytohormones, mainly indol-3-acetic acid (IAA) and other plant-growth regulators (Cassán et al. 2014). Phytostimulation by *Azospirillum* has been mainly linked to root morphological changes resulting in a more developed root system, with a reduction in the main root length and increases in the length of lateral roots, along with more and more branched root hairs (Hadas and Okon 1987, Dobbelaere et al. 1999; Spaepen et al. 2007, Molina-Favero et al. 2008, Cassán et al. 2020). Thus, significant increases in root surface and root volume may be observed (Spaepen et al. 2014).

Recent observations in our laboratory suggested that root architecture changes in *Arabidopsis thaliana* inoculated with *A. argentinense* Az39 are caused by both IAA-dependent and IAA-independent pathways, where *A. argentinense* Az39 flagellin is a key molecule involved in the IAA-independent mechanism (Mora et al. 2023, In Press). These changes ultimately result in an enhanced root-absorbing area, optimizing water uptake and nutrient assimilation, mainly nitrogen. Greater roots might not only improve plant growth but also release more exudates into the rhizosphere (Vives-Peris et al. 2020, Sun et al. 2021), stimulating the growth of other root-associated communities. Thus, *Azospirillum*'s ability to promote root growth can also indirectly impact rhizospheric microbial communities.

Different *Azospirillum* species were isolated from the roots of cereals (e.g. maize, sorghum, rice) and other plants growing in distant parts of the world, such as the US, Africa, Brazil, Canada, and India, among others. *Azospirillum* has also been detected in association with mycorrhizae (Li and Castellano 1987) and diverse and extreme environments, including soil and rhizospheres from arid/semiarid regions (Ilyas et al. 2008, 2012) and saline and alkaline lakes (Hingole and Pathak, 2013) (Table 1).

Overall, these findings demonstrate that one of the most remarkable characteristics of this bacterial genus is its ubiquity. Although usually isolated from plant fractions and thus considered root colonists (Bashan et al. 2004), *Azospirillum* spp. can adapt to highly diverse environments, even polluted or chronically water-stressed. Owing to this notorious adaptability or plasticity and the aforementioned biochemical and physiological features, these bacteria can positively influence the growth of hundreds of plant species, for which they drew attention and became the basis of biological products intended to promote plant growth and increase yields under field conditions, in an environmentally friendly and inexpensive manner. These products are collectively known as "inoculants" or "biofertilizers". In order to be effective, any inoculant (including those based on *Azospirillum*) must comply with minimum quality requirements (Deaker et al. 2011). Among them, the identity of the microorganism intended to act as an "active ingredient" must be corroborated, and appropriate bacterial concentrations to ensure survival and profuse colonization of the target niche must be standardized and guaranteed (Bashan and de-Bashan 2015). In this context, the number of publications related to the tracking of microbial inoculants has increased significantly in the last five years (Manfredini et al. 2021). After offering an overview of the procedures used to detect *Azospirillum* spp., the present review summarizes recent works focusing on *Azospirillum* occurrence in diverse microbiomes and its traceability once released into the environment as an inoculant. The less-known physiological features that may contribute to the remarkable colonization ability that characterizes this bacterium are also briefly discussed.

Procedures to detect and characterize *Azospirillum* spp.

The first *Azospirillum* isolates were identified by culturing soil and plant tissue samples in semi-selective and differential culture media. The procedure, still in use, consists of making serial dilutions and transferring aliquots of these dilutions to appropriate culture media. Some examples of these culture media are provided in Table 1, along with the names of the 27 species recognized to date and information about their ecological and geographical origins.

The most frequent isolation medium used is nitrogen-free semi-solid (NFB), which has no nitrogen and contains malate as the C source (Döbereiner and Day 1976). By including a very low amount of agar, the microaerophilic conditions prevailing some millimeters below the surface create optimal conditions for the nitrogenase activity responsible for BNF in diazotrophic bacteria. The observation of a whitish "halo" strongly suggests its presence.

Some *Azospirillum* species have been identified based on modifications in NFB composition, such as changes in the carbon source or final pH, increased saline concentrations, and the addition of vitamins or other nutritional components (Reis et al. 2015). Alternatively, *Azospirillum* identification has been possible by switching up growth conditions, including the incubation time or temperature (Zhao et al. 2020).

As shown in Table 1, more complex media such as trypticase soy agar (TSA), brain heart infusion (BHI), Reasoner's 2A agar (R2A), or type M agar were necessary to isolate certain members. Moreover, the name *Azospirillum massiliense* appears as *Candidatus* taxa in the CANDIDATUS LIST No. 3, updated to 2020 (Oren and Garrity 2022) and based on the findings by Pagnier et al. (2008), who isolated several new or still uncharacterized genus/species using an *Acanthamoeba polyphaga* co-culture procedure.

Presumptive *Azospirillum* identification is usually based on the colony morphological characteristics in different growth media. Dry, red colonies are typically observed in agar Red Congo, or small and white ones in NFB with bromothymol blue (Cassán et al. 2015).

A negative result on Gram staining followed by observation of fresh smears prepared from young cultures under optical microscopy, to check for cell shape (small bacilli) and motility is generally regarded as confirmatory. Acetylene reduction assays can be carried out to determine if the isolated bacterium has nitrogenase activity, therefore, can accomplish BNF.

If available, scanning electron microscopy (SEM) may also be used. *Azospirillum* spp. appear as pleomorphic curved bacilli with a polar flagellum and cyst-like formations. Differentiation techniques include motility and growth tests in different NaCl concentrations or measurements of pH and temperature ranges (Table 1).

Azospirillum isolates have been identified at the species level through multiple approaches. For instance, the presence of the *nifH* genes-involved in nitrogenase synthesis-may be investigated through PCR amplification (Poly et al. 2001). The

Table 1. *Azospirillum* species reported to date.

Species	Type strain	Culture media	Sample origin	Country	Reference
A. agricola	CC-HIH038	Nutrient agar	Cultivated soil sample	Taiwan	Lin et al. 2016
A. argentinense[*1]	Az39	RC agar	Wheat surface-disinfectant roots	Argentina	dos Santos Ferreira et al. 2022
*A. baldaniorum**	Sp245	Semisolid NFB	Wheat surface disinfected roots	Brazil	dos Santos Ferreira et al. 2020
A. brasilense	Sp7	Semisolid NFB	Rhizosphere of *Digitaria decumbens*	Brazil	Tarrand et al. 1978
A. canadense	DS2	M medium	Corn rhizosphere	Canada	Mehnaz et al. 2007a
A. cavernae	K2W22B-5	R2A agar	Water samples	China	Zhu et al. 2021
A. doebereinerae	GSF71	Semisolid NFB + biotin	Washed roots of *Miscanthus*	Germany	Eckert et al. 2001
A. fermentarium	CC-LY743	Nutrient agar	Fermentative tank	Taiwan	Lin et al. 2013
A. formosense	CC-Nfb-7	NFB agar	Agricultural soil	Taiwan	Lin et al. 2012
A. griseum	L-25–5 w-1	R2A agar	Water at Baiyang Lake	China	Yang et al. 2019
A. halopraeferens	Au 4	Semisolid NFB + 1.5% NaCl	Rhizoplane *Leptochloa fusca* (L.)	Pakistan	Reinhold et al. 1987
A. himalayense	ptl-3	Jensen's agar	Rhizosphere maize plant	India	Tyagi and Singh 2014
A. humicireducens	SgZ-5	MSM	Microbial fuel cell (MFC)	China	Zhou et al. 2013
A. largimobile	ACM 2041	LWA	Fresh water	Australia	Skerman et al. 1983
A. lipoferum	4B	Semisolid NFB	Wheat roots	Brazil	Tarrand et al. 1978
A. melinis	TMCY 0552	LGI and NFB medium	Stems and roots from *Melinis minutiflora*	China	Peng et al. 2006
A. oleiclasticum	RWY-5–1-1	R2A medium	Oil mixture Yuman Oilfield	China	Wu et al. 2021
A. oryzae	COC8	M medium	Root of rice plant	Japan	Xie and Yokota, 2005
A. palustre	B2	Semisolid NFB	*Sphagnum* samples	Russia	Tikhonova et al. 2019
A. picis	IMMIB TAR-3	Nutrient agar	Discarded road tar	Taiwan	Lin et al. 2009
A. ramasamyi	M2T2B2	R2A agar	Fermented bovine products	Korea	Anandham et al. 2019
A. rugosum	IMMIB AFH-6	Nutrient agar	Oil-contaminated soil	Taiwan	Young et al. 2008
A. soli	CC-LY788	Nutrient agar	Agricultural soil	Taiwan	Lin et al. 2015
A. tabaci	W712	R2A agar	Rhizosphere soil of *Nicotiana tabacum*	China	Duan et al. 2022
A. thermophilum	CFH 70 021	T5 medium	Hot spring soil sample	China	Zhao et al. 2020
A. thiophilum	BV-S	Semisolid MPSS + FeS + Vitamins	Bacterial mat of sulfide spring	Russia	Lavrinenko et al. 2010
A. zeae	N7	M medium	Corn rhizosphere	Canada	Mehnaz et al. 2007b

NFB: nitrogen free; RC: Congo red; R2A: Reasoner's 2A; MSM: Mineral salts medium; LWA: Lake water agar
*Genome-based reclassification

ability to produce phytohormones can also be explored (dos Santos Ferreira et al. 2022).

As generally applicable to any other bacterial isolate, further characterization of *Azospirillum* isolates may be achieved through different techniques. Among them, it may be mentioned the analysis of fatty acids and respiratory quinones composition by gas chromatography (GC) and 2D thin layer chromatography (2D-TLC), respectively, of protein patterns through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), or of carbon sources utilization and enzymatic/biochemical properties through automated systems such as API 20 NE and Biolog GEN III (Young et al. 2015). Mainly for research purposes (e.g. identification of new species) and assuming access to molecular techniques, the 16S rRNA gene can be sequenced, and phylogenetic studies may be performed. Additional information may be obtained by fingerprint techniques such as random amplification of polymorphic DNA (RAPD) or denaturing gradient gel electrophoresis (DGGE) (Lin et al. 2011, Ilyas et al. 2012). Despite the wide array of available methodologies, unequivocal classification of *Azospirillum* species is only achieved through complete genome sequencing and subsequent comparative analyses. The genome sequence provides an estimation of overall genome relatedness indices (OGRIs), such as DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI).

Azospirillum: a wandering microbe?

Undoubtedly, metagonomics has opened new roads in many biological disciplines, including microbial ecology. Apart from being isolated from various habitats through classic microbiological procedures, in recent years, *Azospirillum* has been detected as part of animal, human, and environmental microbiomes through metagenomics and other molecular approaches. The following sections summarize these recent findings.

Azospirillum in microbiomes from animals

The vast number of metagenomic studies addressing human health rarely identified *Azospirillum*. However, *Azospirillum*

DNA has been detected in fecal samples from patients responding favourably to nivolumab, a monoclonal antibody used in cancer treatment, by amplifying and sequencing V3–V4 regions of the 16S rRNA bacterial gene at months 0 and 2 after treatment (Chung et al. 2021). Shotgun metagenomic sequencing of stools from pre-school-aged children in Zimbabwe revealed that the relative abundance of certain bacteria and fungi taxa differed between schistosome-infected and uninfected children. Increases in the relative abundance of bacteria such as *Pseudomonas, Stenotrophomonas, Derxia,* and *Thalassospira*, and decreases in *Azospirillum*were linked to the absence of the parasite *Schistosoma haematobium*, the causal agent of schistosomiasis (Osakunor et al. 2020).

These observations suggest that certain *Azospirillum* members might be part of a healthy gut microbiome; however, they have also been identified in the sputum of severe asthmatic patients (Wang et al. 2021). On the other hand, Wang et al. (2018) examined the effect of heat stress on broiler chicken's gut microbiome using pyrosequencing technologies and detected enrichment in *Clostridium*, *Streptophyta*, *Oscillibacter*, *Faecalibacterium*, *Rothia*, and *Azospirillum* in heat-stressed animals, while other bacteria including *Coprococcus* and *Streptococcus* were reduced. Presumably, *Azospirillum* might 'make their way' into both human and farm animal microbiomes after being inoculated on crops destined to human or animal consumption. However, further evaluation directed to understand the features allowing these microbes to succeed in colonizing these less-recognized habitats, and the kind of impact they might have on the resident microbiota, are still lacking.

Also, *Azospirillum* members have been identified through metagenomic procedures in different aquatic habitats and microbiomes. This includes mucosa samples from the Eurasian carp (*Cyprinus carpio*) (Meng et al. 2021) and human remains (bones) from the seabed (Kim et al. 2020).

Azospirillum in microbiomes from aquatic environments

Azospirillum was detected in samples from the sea sponge *Lamellodysidea herbacea* (Podell et al. 2020) and within microbial communities associated with seagrasses such as *Halophila ovalis,* where their relative abundance was correlated with 100% exposure to irradiance under normal light conditions (Martin et al. 2018). Despite usually inhabiting terrestrial plants, the presence of *Azospirillum* spp. in marine environments makes sense given their evolutionary origins: practically all their close relatives are aquatic (Wisniewski-Dyé et al. 2012).

Azospirillum in plant and soil microbiomes

So far, most research on *Azospirillum* has focused on soils, where these bacteria may be part of the native microbiota associated with certain plant species or linked to specific agricultural practices. For instance, a HiSeq-based community structure study revealed that *Azospirillum* was among the top 20 bacterial genera in greenhouse soil samples subjected to reductive soil disinfection (RSD) (Yanlong et al. 2021), a technique primarily intended to reduce soil-borne pathogens by stimulating microbial anaerobic degradation of added labile carbon (straw, in this case) through flooding. Additionally, metagenomic analyses detected *Azospirillum* after organic fertilizers application, including vermicompost and cow manure (Li et al. 2020), and in the rhizospheric microbiomes of coffee plantations under intensive farming or transitioning from intensive to organic farming (Caldwell et al. 2015).

Pyrosequencing used to determine root endophytic community in rice plants either uninoculated and fertilized with urea or inoculated with *Rhizobium leguminosarum* revealed enrichment in several diazotrophic rhizobacteria including *Azospirillum* (Jha et al. 2020).

Zhang et al. (2021) analyzed maize-associated microbial communities at different distances (0–0.5, 0.5–1, 1–2, 2–4, and 4–9 cm) away from the root surface in the rhizosphere, and detected enrichment in *Azospirillum*, along with other genera such as *Sphingomonas*, *Sphingobium*, *Pseudolabrys*, and *Novosphingobium*, at 0.5 cm from the root in plants fertilized with nitrate.

The microbiomes of soils previously cultivated with rapeseed (*Brassica napus*), pea (*Pisum sativum* L. *ssp. Arvense*), and wheat (*Triticum aestivum* L.) in a district of north-eastern Poland all revealed *Azospirillum* among the dominant genera (those with a number of readings >1% of all OTUs), with a higher relative abundance after culturing wheat compared to rape and pea (Wyszkowska et al. 2019).

Some authors have suggested that *Azospirillum* abundance in the rhizosphere microbiome might be correlated with the plant's ability to recruit in its root system certain bacterial species under adverse environmental or physiological conditions (Chaparro et al. 2012). Interestingly, Wang et al. (2020) reported a lower relative abundance of *Rhizobiaceae*, *Lysobacter antibioticus*, and *Bradyrhizobium japonicum* and a higher relative abundance of *A. lipoferum* and *A. brasilense* in rhizospheric soil samples of sick ramie (*Boehmeria nivea* L. Gaud) than in those collected near healthy plants.

In light of these observations, it may be speculated that the composition of root exudates released by plants subjected to stress might encourage the selective recruitment of *Azospirillum* and other bacteria to the rhizosphere, increasing their prevalence (Wang et al. 2020).

In another interesting study (Kudjordjie et al. 2021), the roots of some *A. thaliana* mutant lines disrupted in metabolic pathways for the synthesis of glucosinolates, flavonoids, and other relevant defense-signaling compounds were sampled, and genome sequencing (Illumina MiSeq platform) was performed to obtain bacterial and fungal community profiles. These mutations specifically enriched or depleted microbial taxa. Compared to the parental lines, mutant lines were mostly enriched in several fungal and bacterial genera, OTUs assigned to the genera *Nocardioides* and *Azospirillum* were the most highly enriched in several GLS mutants. Other mutant lines were enriched in *Fluviicola*, *Azospirillum,* and *Flavobacterium*. These observations suggest that the rhizosphere may become richer in *Azospirillum* and other 'recruitable' microorganisms under certain environmental/physiological conditions, mainly due to plant defense mechanisms and an altered root exudation pattern.

Silva et al. (2022) provided metagenomic evidence in favour of this notion through a study intended to compare conventional versus organic agricultural practices in tomato in relation to the incidence of the root-knot nematode *Meloidogyne incognita*. These authors observed that root-knot nematode-containing rhizospheres (due to having received the organic amendment) recruited nematode-antagonistic bacteria and fungi more efficiently than those under conventional management. *Pseudomonas, Serratia, Bradyrhizobium, Burkholderia*,

and *Azospirillum* were some of these antagonistic bacteria with higher relative abundance.

Numerous reports have highlighted *Azospirillum*'s ability to survive and mitigate abiotic stresses while interacting with plants (Fukami et al. 2017, 2018, Molina et al. 2018). Metagenomic analyses performed in the rhizosphere of three halotolerant plants (*Reaumuria songarica*, *Nitraria tangutorum*, and *Alhagi sparsifolia*), all three growing profusely in the Junggar sedimentary basin of China, confirmed that the relative abundance of *Azospirillum* and *Bradyrhizobium* did not vary under water stress and that this might be connected to the plants' survival strategy (Li et al. 2022a). Likewise, *Azospirillum* members were identified in the microbiome of Pequin pepper plants (*Capsicum annuum* var. *glabriusculum*) grown in arid areas under extreme water stress.

In soil samples collected in the tropical savanna of the Brazilian 'Cerrado', *Azospirillum* was identified either in samples of soils under conventional management or subjected to conservation tillage; however, its relative abundance was higher in undisturbed soil samples (Souza et al. 2016).

In a greenhouse experiment addressing the possible effects of triazole fungicides (foliar application) on barley-associated soil community (Baćmaga et al. 2020), *Bacillus arabhattai*, *Bacillus soli*, and *Bacillus simplex* were detected exclusively in control samples, whereas *Ramlibacter tataounensis*, *Azospirillum palatum*, and *Kaistobacter terrae* were exclusively found in treated samples. Likewise, microbiome analyses revealed enrichment in *Azospirillum* and other genera (e.g. *Herbaspirillum*, *Sphingomonas*, *Caulobacter*, and *Brevundimonas*) in an indigenous agricultural soil in Taiwan exposed to hexabromocyclododecane (a persistent organic pollutant), linking these changes in microbiomes to microbial abilities to degrade or biotransform complex and recalcitrant compounds into less toxic compounds (Li et al. 2022b).

Some *Azospirillum* members could thus play a crucial role in bioremediation/assisted phytoremediation schemes, not only by promoting plant growth and mitigating abiotic stress but also by contributing to the degradation/attenuation of the polluting agents (Kaur et al. 2021).

Other metagenomic studies also revealed a considerable relative abundance of *Azospirillum* in aerosols emitted from the Amazon rainforest, but only during the dry season (Souza et al. 2021). Based on sequence comparisons with *Azospirillum* reference genomes, these authors concluded that the three most abundant OTUs matched tentatively *A. brasilense*, *A. lipoferum*, and *A. oryzae*, and suggested as their possible origin the phyllosphere of plants growing in the surroundings during that season.

In Spain, Mediavilla et al. (2019) carried out soil DNA metabarcoding in *Cistus ladanifer* scrublands destined to produce the edible mushroom *Boletus edulis*. Their goals were to analyze the impact of site history management and fire prevention treatments on bacterial richness and community composition and to link these findings with *B. edulis* productivity. They detected greater effects for the former and found that *Azospirillum* (Proteobacteria), *Gemmatimonas* (Gemmatimonadetes), and *Opitutus* (Verrucomicrobia) could be considered markers of the most productive sites for sporocarp formation. This finding suggests that *Azospirillum* may interact with *B. edulis* enhancing its fructification.

Summing up, *Azospirillum* can associate with many different crops, but this ability seems to be modulated by the type of agriculture practiced and the environmental conditions prevailing.

The *'Azospirillum* paradox'

Despite its great versatility and identification in heterogeneous environments, *Azospirillum* is rarely mentioned or referenced in the metagenomic analyses performed in soil and plant samples after inoculation. One possible explanation for this paradox may be related to the relatively low number of *Azospirillum* members in the soil compared to other bacterial genera, making their molecular identification more difficult.

By contrast, *Azospirillum* isolation through classic microbiological techniques has been frequently reported, suggesting that those procedures can be quite simple for laboratories with appropriate experience in handling these bacteria. Several reports tend to corroborate this idea. Qaisrani et al. (2019) compared metagenomic analyses involving culture-dependent and culture-independent methodologies. Using the former, they were able to isolate *Azospirillum* from maize rhizosphere, but they did not find the expected 16S rRNA or *nifH* gene sequences. Coniglio et al. (2022) studied the rhizospheric microbiome of maize inoculated with *A. argentinense* Az39 (formerly *A. brasilense* Az39) and compared the results with those obtained in uninoculated plants and bulk soil: they detected these bacteria only in the rhizosphere of inoculated plants.

Renoud et al. 2022a, 2022b) were unable to find the inoculated strain *A. lipoferum* CRT1 in the rhizosphere of maize through specific q-PCR, and both the six-leaf stage and the flowering stage proved equally unfruitful in this respect. Similar results were obtained by Urrea-Valencia et al. (2021) in maize inoculated with *A. brasilense* Ab-V5 and Ab-V6. However, Matsumura et al. (2015), Coniglio et al. (2022), Preepremmot et al. (2020) found the inoculated bacteria in samples collected from maize and rice rhizospheres. Matsumura et al. (2015) reported that inoculated *Azospirillum* strains could be detected when nitrogen was added at concentrations below normal fertilization levels but not under normal fertilization rates.

Now, new sequencing and sequence analysis techniques allow extracting information from genetic material obtained from diverse environmental samples, and microbes' detection has become more likely even if the target group or genus is not very abundant (Durazzi et al. 2021). On the other hand, protecting *Azospirillum* from abiotic and biotic stresses (de Bashan and Bashan 2008, Santos et al. 2020, Takahashi et al. 2022) by developing new carriers might increase their survival in the soil and, therefore, their detection. In this regard, it should be said that *Azospirillum* is mainly supplied in peat or liquid inoculant formulations. These delivery methods do not protect cells from eventual stressing conditions (Urrea-Valencia et al. 2021, Takahashi et al. 2022). Bacteria encapsulation using biodegradable, non-expensive, and non-toxic biopolymers as carriers proved to be a sustainable alternative to extend *Azospirillum* span-life in the environment, thus favouring the performance of these bioproducts (Bashan et al. 2002, Lima-Tenório et al. 2023).

More advanced sequencing technologies and sequence analysis tools will probably overcome this paradox. On the other hand, detection through RNA sequencing would allow a more comprehensive approach by targeting only metabolically active cells. More sensitive and accurate methods for

detecting these ubiquitous but elusive bacteria are needed, not only to monitor their fate once released into the environment but also to understand their behavior and lifestyle in the lithosphere.

Effects of *Azospirillum* inoculation on plant microbial communities

Until recently, methodological limitations prevented us from getting a detailed picture of microbial communities in a given environment. Therefore, there is still scarce information on the impact of *Azospirillum* introduction into the plant-soil system and their established microbial communities.

Data collected up to now have ranged from none or very few observable effects (Herschkovitz et al. 2005, Lerner et al. 2006, Pedraza et al. 2009) to many positive effects on community structure (Correa et al. 2006, Baudoin et al. 2009, Naiman et al. 2009). This variability was generally attributed to differences in environmental conditions and plant species. Studies based on 16S rRNA sequencing indicate that the ability of an inoculated bacterium to promote plant growth in a given environment is inversely proportional to the degree of disturbance it causes in the existing microbial population (Renaud et al. 2022b). Therefore, the most efficient plant-growth promoters are not expected to cause significant changes in the microbial communities associated with the target plant. Still, modifications in microbial community composition upon *Azospirillum* introduction, with impact on specific bacterial groups, have been communicated.

For example, da Costa et al. (2018) compared the microbial composition in the soil before maize cultivation with that of the rhizosphere in *Azospirillum*-inoculated maize. They noticed a link between inoculation and the presence of members from *Comamonadaceae* family, Betaproteobacteria, *Pseudonocardia*, and *Micrococcaceae*.

Azospirillum inoculation was also associated with the absence of *Enterobacteriaceae* members (da Costa et al. 2018). Coniglio et al. (2022) found a positive association between maize inoculation with *A. argentinense* Az39 and the presence of *Pseudomonas*, *Burkholderia*, *Massilia*, *Sphingobium*, and *Rhizobium* in the rhizosphere.

Bao et al. (2013) found that rice inoculation with *Azospirillum* sp. B510 influenced minority but not majority groups in the shoot microbiome. Likewise, after maize co-inoculation with *Burkholderia ambifaria* RZ2MS16 and *A. brasilense* Ab-V5, certain groups in the microbiome became more abundant, particularly bacteria belonging to *Actinobacteria* class and *Actinomycetales* order (Ferrarezi et al. 2022). Then, the specific microbial groups reported to be altered due to *Azospirillum* inoculation vary considerably from one report to the other. Differences might result from diverse soil origins and different bacterial strains and crop cultivars assayed, and perhaps also from heterogeneous sampling times and environmental conditions.

Changes in the relative abundance of specific microbial groups after *Azospirillum* inoculation were also analyzed through metagenomic approaches. In southeast France, Florio et al. (2017) assessed the effect of maize inoculation with *A. lipoferum* CRT1 on nitrifying and denitrifying microorganisms, as well as on ammonia oxidizers (both Bacteria and Archaea), under C-limiting and C-sufficient conditions. The effects on the nitrification process and the abundance of nitrifying microorganisms differed across sampling dates, sites, and nutrient availability. Another set of studies performed in three French fields aimed at investigating how maize inoculation with *A. lipoferum* CRT1 affected rhizospheric communities with BNF activity, aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, and 2,4-diacetylphloroglucinol production (Renoud et al. 2022a), and found that inoculation modified the composition of the diazotrophic community in the second year; the group of ACC deaminase producers changed to a lesser extent. A follow-up study by the same research group revealed as a relevant variable the dose applied (Renoud et al. 2022b).

Overall, the evidence gathered through modern massive sequencing methods demonstrates that plant inoculation with *Azospirillum* spp. may alternative microbial communities regarding taxonomic composition and functionality. These changes depend on multiple factors, including the strain inoculated, the location of the cultivated area, the cultivation conditions, the bacterial concentration in the inoculant, and the growth stage of the target crop. Although the community composition seems not to be significantly modified by *Azospirillum* introduction, some minority groups previously described as plant-growth promoters, either individually or when co-inoculated with *Azospirillum*, may be favoured. This includes *Pseudomonas*, *Burkholderia*, and *Rhizobium* (Ferrarezi et al. 2022).

Sharing ecological niches

Metagenomics studies have also made it possible to determine those microorganisms that usually occupy the same ecological niche colonized by *Azospirillum*. Matsumura et al. (2015) identified *Azospirillum* and *Rhizobium* as part of the endophytic population in stem samples of maize fertilized with low nitrogen concentrations and inoculated with *A. brasilense* Ab-V5. The simultaneous finding of these genera has also been documented in the rhizosphere of maize, rice, and *Nicotiana benthamiana*, as well as in pristine soils (Souza et al. 2016, Jha et al. 2020, Coniglio et al. 2022, Liu et al. 2022).

Coniglio et al. (2022) recorded the existence of *Pseudomonas* after maize inoculation with *A. argentinense* Az39. Both *Azospirillum* and *Pseudomonas* have been detected together in soils exposed to hydrocarbon contamination (Ruiz et al. 2021) and in vermicompost consisting of vegetable waste, cow manure, and mud (Li et al. 2020). *Bradyrhizobium* was identified alongside *Azospirillum* in samples taken from the rhizosphere of trees (Kaur et al. 2021), in vermicompost (Li et al. 2020), and in the rhizosphere of plants growing under arid conditions (Li et al. 2022a). The finding of these genera sharing the same ecological niche suggests that they may function in a complementary way to promote plant growth.

Traceability of *Azospirillum* strains used as inoculants in agriculture

Several methods are available to accurately identify, quantify, track, and monitor *Azospirillum* populations in seeds, plants, and soils (Table 2). These methods may be divided into nucleic acid-based, reporter gene-based, and immunological reaction-based (Rilling et al. 2019).

Indirectly, these methods may also provide information about *Azospirillum*'s lifestyle under different environmental conditions. At the same time, the lifestyle of each *Azospirillum* strain (endophytic, rhizospheric, and phyllospheric) and

Table 2. Summary of reports about *Azospirillum* traceability after inoculation.

Strain	Assay type	Method	Crop/Product	Organ/Tissue	Time	Quantity	Reference
Nuclei acid-based							
A. lipoferum CRT1	Field	Hybridization (16SrRNA)	Maize	Root	40 DAI 140 DAI	1e + 8 CFU.g^{-1} 1e + 4 CFU.g^{-1}	El Zemrany et al. 2006
A. lipoferum CRT1	*in vitro* Field	qPCR ss (SCAR)	Maize	Root rhizosphere	10 DAI 18 DAI	1e + 6 eqCFU. g^{-1} 1e + 4 eqCFU.g^{-1}	Couillerot et al. 2010a
A. brasilense FP2	*in vitro* Greenhouse	qPCR gs (*nif*A)	Maize	Root	1 DAI 4 DAI 7/10 DAI 1/4/7 DAI 10 DAI	1e + 6 DNA CN.g^{-1} 1e + 8 DNA CN.g^{-1} 1e + 9 DNA CN.g^{-1} 1e + 8 DNA CN.g^{-1} 1e + 7 DNA CN.g^{-1}	Faleiro et al. 2013
A. brasilense UAP-154 *A. brasilense* CFN-535	*in vitro*	qPCR ss (SCAR)	Maize	Root rhizosphere	10 DAI	1e + 6 eqCFU.g^{-1} 1e + 5 eqCFU.g^{-1}	Couillerot et al. 2010b
A. brasilense CFN-535 *A. brasilense* UAP-154 *A. lipoferum* CRT1	*in vitro*	qPCR ss (SCAR)	Maize	Rhizosphere	10/35 DAI	1e + 5/7 eqCFU.g^{-1} 1e + 4/6 eqCFU.g^{-1} 1e+</6 eqCFU.g^{-1}	Couillerot et al. 2013
A. brasilense FP2	*in vitro*	qPCR ss (SCAR) qPCR sps (SCAR)	Wheat	Root	1/13 DAI	1e + 7/8 eqCFU.g^{-1} 1e + 7/8 eqCFU.g^{-1}	Stets et al. 2015
A. lipoferum Az204 *A. brasilense* Sp7	*in vitro*	qPCR ss (SCAR)	Inoculant	-	-	-	Reddy-Priya et al. 2016
A. brasilense Sp7	*in vitro*	qPCR ss (SCAR)	Inoculant maize	- Rhizosphere	15/60 DAI	1e + 4 eqCFU.ml^{-1} 1e + 5/6 eqCFU.g^{-1} 1e + 4 eqCFU.g^{-1}	Reddy-Priya et al. 2018
A. brasilense Sp7	*in vitro*	qPCR gs	Common bean	Root stem leaf seed	V, ER, M	Bacteria/plant cell	Malinich and Bauer 2018
A. brasilense Ab-V5 *A. brasilense* Ab-V6	*Field*	qPCR sps (SCAR)	Maize	Rhizosphere root	1/7 DAI 11 DAI 13/75 DAI	1e + 5 eqCFU.g^{-1} 1e + 5 eqCFU.g^{-1} 1e + 3 eqCFU.g^{-1} 1e + 4 eqCFU.g^{-1}	Urrea-Valencia et al. 2021

Table 2. Continued

Strain	Assay type	Method	Crop/Product	Organ/Tissue	Time	Quantity	Reference
A. brasilense FP2	*in vitro*	qPCR ss (SCAR/PMA)	Maize	Root endophytes	7 DAI	1e + 6 eqCFU.g^{-1}	da Cunha et al. 2020
A. baldaniorum Sp245	Field	qPCR ss (SCAR)	Brachiaria	Root shoot	60 DAI	1e + 3 eqCFU/g.$^{-1}$ 1e + 2 eqCFU/g.$^{-1}$	Soares et al. 2021
A. brasilense FP2	*in vitro*	qPCR ss + CFU (SCAR)	Wheat	Seed root	48 HAI 21 DAI	1e + 6 CFU g^{-1} 2e + 6 eqCFU g^{-1}	Takahashi et al. 2022
Azospirillum sp. B510	*in vitro*	CRISPR (loci-PCR)	Wheat	Root	14 DAI	Qualitative	Rilling et al. 2021
A. lipoferum CRT1	Field	qPCR ss (SCAR)	Maize	Root rhizosphere	SL-F	Absent	Renoud et al. 2022a
A. lipoferum CRT1	Field	qPCR ss (SCAR)	Maize	Root rhizosphere	SL-F	Absent	Renoud et al. 2022b
A. argentinense Az39	*in vitro*	PCR (end point)	Inoculants	-	-	-	Coniglio et al. 2020
A. amazonense CBAmC	*in vitro*	FISH + MPN	Sugarcane	Root endophytes	5 DAI	4.4e + 4 CFU.g^{-1}	Oliveira et al. 2009
A. brasilense Sp7 *A. baldaniorum* Sp245	*in vitro*	FISH + CFU	Wheat	Root	4/7 WAI	1e + 4 CFU.g^{-1}	Rothballe et al. 2003
Immunological reaction-based							
A. baldaniorum Sp245	*in vitro*	Immunoblot	Rice	Soil	1 HAI	4.4e + 8 CFU.g^{-1}	Krishnen et al. 2011
A. brasilense Sp7	*in vitro*	CIA	Wheat	Rhizosphere	42 DAI	1e + 5 CFU.mg^{-1}	Schloter et al. 1992
A. brasilense Sp7 *A. brasilense* Wa5 *A. baldaniorum* Sp245	*in vitro*	ELISA-Mab	Wheat	Root Root endophytes	4/6/8/10/14 WAI	1e + 5/6 CFU.g^{-1} 1e + 4/5 CFU.g^{-1} 1e + 5/7 CFU.g^{-1}	Schloter and Hartmann 1998
A. baldaniorum Sp245	*in vitro*	ELISA-indirect	Wheat	Root	1 DAI	-	Yegorenkova et al. 2016

Table 2. Continued

Strain	Assay type	Method	Crop/Product	Organ/Tissue	Time	Quantity	Reference
Reporter gene-based							
A. brasilense Sp7 *A. brasilense* 7067	*in vitro*	*lacZ-nifA* + MPN	Wheat	Root	10 DAI	2.7e + 7 $CFU.g^{-1}$ 1.9e + 7 $CFU.g^{-1}$	Arsène et al. 1994
A. brasilense Cd	*in vitro*	*lacZ-nifA* + MPN	Wheat	Root endophytes	14 DAI	1e + 5/7 $CFU.g^{-1}$ 1e + 8/9 $CFU.g^{-1}$	Fischer et al. 2000
A. brasilense FP2	*in vitro*	*gus/gfp*	Wheat	Root	15/30 DAI	*Not counted*	Ramos et al. 2002
A. brasilense Cd	*in vitro*	*gusA** + CFU	Tomato	Endophytes stem leaf	30 DAI	3.9e + 7 $CFU.g^{-1}$ 1.6e + 5 $CFU.g^{-1}$ 1.6e + 5 $CFU.g^{-1}$	Botta et al. 2013
A. brasilense FP2 *A. brasilense* HM053	*in vitro*	*gusA-nifH* + CFU	Wheat	Root	4 DAI	1.0e + 7 $CFU.g^{-1}$ 1.5e + 7 $CFU.g^{-1}$	Santos et al., 2017a
A. brasilense FP2 *A. brasilense* HM053	*in vitro*	*gusA-nifH*	Barley	Root	3/7/12 DAI	*Not counted*	Santos et al., 2017b
A. brasilense Ab-V5 *A. brasilense* Ab-V6	*in vitro*	*egfp/eyfp* + CFU	Maize	Leaf	1/2 DAI	1e + 2/5 $CFU.cm^{-2}$ 5e + 2/5 $CFU.cm^{-2}$	Fukami et al. 2017
A. brasilense Sp7	*in vitro*	*Gfp* + CFU (rif^r)	Common bean	Root Stem Leaf Seed	ER	1e + 3 CFU. g^{-1} 1e + 1 CFU. g^{-1} 1e + 2 CFU. g^{-1} 1e + 2 CFU. g^{-1}	Malinich and Bauer 2018
A. brasilense 1224	*in vitro*	*gus/gfp; mCherry*	Onion	Root Bulb (stem)	0/3/7/28 DAI	*Not counted*	Hong et al. 2019
A. argentinense Az39	*in vitro*	*dsRed** + CFU	Soybean	Leaf	1 DAI	2.9e + 3 $CFU.g^{-1}$	Puente et al. 2021
A. brasilense Sp7	*in vitro*	*gfp**	Alfalfa	Root	5 DAI	**	O'Neal et al. 2020

*constitutive; **maintained or increased in relation to the inoculation

References: HAI: hours after inoculation; DAI: days after inoculation; WAI: weeks after inoculation; ER: early reproductive stage; V: vegetative stage; M: mature stage; SL: six-leaf stage; F: flowering stage; MPN: most probably number; Mab: monoclonal antibodies; ss: strain specific; gs: genus specific; sps: species specific; CIA: chemoluminescence imunoasssay; CFU: colony forming units counting; MPN: most probably number; CN: copy number

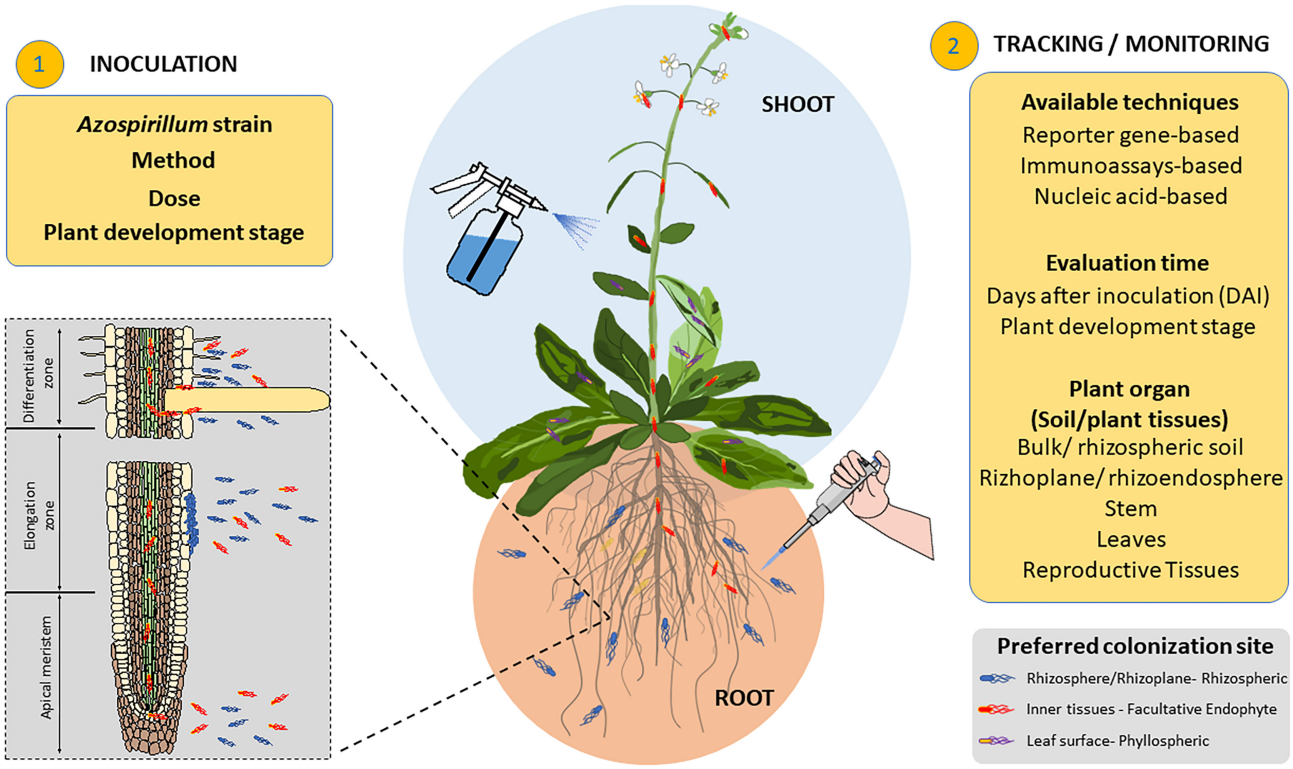

Figure 1. Factors influencing *Azospirillum* inoculation efficacy and strategies for its tracking and monitoring. Tracking methods may be more successful if the preferred colonization site of the inoculated strain is known and better carriers, able to protect *Azospirillum* cells from biotic and abiotic stressors, are used in commercial formulations.

the colonization ability must be considered when choosing the best inoculation and tracking methods (Fig. 1).

For example, a rhizospheric bacteria with good colonization capacity can be inoculated on the seeds or to the soil, whereas strains more adapted to the phyllosphere should be rather applied as a leaf spray. In this way, inoculating *Azospirillum* close to its target niche would increase the chance of successful colonization and, therefore, of plant-growth promotion. In this sense, bacteria with a greater ability to adapt to different niches (i.e. systemic facultative endophytes) will enable a greater spectrum of inoculation methods, which constitutes an agronomic advantage.

Even when the variable results regarding plant growth promotion by inoculated *Azospirillum* have been extensively documented, few studies have attempted to correlate *Azospirillum* colonization efficiency with growth promotion performance. Some pioneer studies in the 1990s using immunological reaction-based assays allowed the detection of *A. brasilense* Sp7, *A. brasilense* Wa5, and *A. brasilense* Sp245 (now *A. baldaniorum* Sp245) in wheat roots (Schloter and Hartmann 1998). This study proved the endophytic behavior of the last strain, while *A. brasilense* strains were unable to colonize the inner tissues. Later, with the advent of reporter genes, it was possible to recognize the preferential colonization regions of many *Azospirillum* strains.

Through *gusA-nifH* fusion, Santos et al. (2017b) observed that *A. brasilense* Sp7-derivative strains FP2 and HM053 colonized mainly the emerging points of the lateral roots and the root hair zone of wheat. Similar results were obtained in alfalfa and barley (Santos et al. 2017a, O'Neal et al. 2020). Studies using reporter genes also allowed tracking tissue colonization by *Azospirillum* after applying this inoculant as a leaf spray on maize (Fukami et al. 2017) and soybean (Puente et al. 2021) phyllosphere. To identify *Azospirillum* at the genus level, Lin et al. (2011) performed PCR with primers targeting a hypervariable region in the 16S rRNA.

Identification was thus achieved on the basis of DNA sequencing from pure and complex cultures. On the other hand, the *ipdC* gene sequence was used to differentiate between strains of *A. lipoferum* and *A. brasilense* (Jijón-Moreno et al. 2015). This gene allowed distinguishing microorganisms within the *Azospirillum* genus accurately, making it possible to detect them in complex samples after being inoculated, thus contributing to a better understanding of the bacterium lifestyle in the environment.

Malinich and Bauer (2018) used the primers designed by Lin et al. (2011) to confirm the identity of *Azospirillum* isolated from inoculated *Phaseolus vulgaris* (common bean) at different phenological stages. The specific genus primers were combined with qPCR, transcriptomics technologies, confocal microscopy, and culturing techniques to track the bacteria throughout the plant's life cycle. This multimethodological approach revealed that *Azospirillum* colonizes bean tissues profusely and is vertically transmitted to the next generation through the seeds (Malinich and Bauer 2018).

Additionally, fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy allowed *Azospirillum* detection as an endophyte in wheat under optimal and salt-stressed conditions 30 days after inoculation (Rothballer et al. 2003, Nabti et al. 2010). Using FISH, it was also demonstrated that *Azospirillum* could survive and colonize sorghum plants in desert soil when co-inoculated in alginate beads with *Chlorella sorokiniana* (Trejo et al. 2012, Lopez et al. 2013).

The complexity posed by combining many methods may be overcome by qPCR techniques, which enable the detection of specific strains. Fancelli et al. (1998) were the first to search specifically for *Azospirillum* spp. using a probe obtained from DNA fragments generated through RAPD. The probe was successful in detecting *A. lipoferum* ATCC29731 in sorghum roots 24 hours after inoculation. An article published in 2006 describes the use of strain-specific probes to detect inoculated *A. lipoferum* CRT1 in maize roots in the field by targeting the 16S rRNA at hybridization (Table 2). This strain was detected for up to 140 days after inoculation, at around 1e + 5 CFU.g^{-1} (El Zemrany et al. 2006). That probe was used to confirm the existence of colonies that had been previously counted and may have derived from phylogenetically related microorganisms.

Several *Azospirillum* strains were identified and quantified through qPCR in samples obtained from maize tissues and rhizospheric soil. These bacteria could be detected in maize rhizosphere under lab conditions through strain-specific qPCR between 10 and 60 days after inoculation (Couillerot et al. 2010a,b). This technique also allowed the detection of inoculated *A. brasilense* FP2 in wheat roots (Stets et al. 2015) and inoculated *A. brasilense* Sp7 in maize rhizosphere (Reddy-Priya et al. 2018). However, other authors failed to detect the inoculated *Azospirillum* strains for long periods (Urrea-Valencia et al. 2021, Renoud et al. 2022a, 2022b). Regarding the detection of inoculated bacteria in maize tissues, the strains introduced were detectable on the first days after inoculation and long after that in root samples (Soares et al. 2021, Urrea-Valencia et al. 2021, Takahashi et al. 2022).

In maize roots, the population of *A. brasilense* elite strains Ab-V5 and Ab-V6 seems not to undergo great variation along plant development, except for the V2 stage, when the population decreased to 1e + 3 CFU g^{-1} (Urrea-Valencia et al. 2021). A significative drop in the number of eqCFU of *A. brasilense* FP2 was also reported in the initial vegetative stage of wheat (Takahashi et al. 2022). Through qPCR studies, it was demonstrated the viability of some *Azospirillum* strains in commercial inoculants and maize roots for up to 7 days after inoculation when the samples were pre-treated with propidium monoazide (PMA) (da-Cunha et al. 2020).

These results indicate that *Azospirillum* presence in the rhizosphere may be ephemeral. However, those bacteria that manage to survive do establish in association with the host plant and promote its growth and development. This enhances yield and might also modify, to some extent, the native microbial communities. Beyond the purpose of tracking *Azospirillum* in the environment, some extremely efficient methodologies have been designed to identify strains in commercial products and thus guarantee product quality prior to inoculation (Reddy-Priya et al. 2016, Reddy-Priya et al. 2018, Coniglio et al. 2020).

As evidenced by the reports summarized in this section, *Azospirillum* is a versatile and ubiquitous genus with great biotechnological potential.

What is new about functional characteristics associated with *Azospirillum*'s rhizospheric lifestyle?

The colonization of plant roots by *Azospirillum* has been suggested to occur over two stages. The first one is considered to be flagellum-mediated and reversible (Croes et al. 1993); the second one is irreversible and involves exopolysaccharides (EPSs) production and the formation of cell aggregates (Michiels et al. 1991).

Cell motility and biofilm formation have also been linked to the colonization of plant tissues by *Azospirillum* (Burdman et al. 2008), as well as chemotaxis and the interaction with surrounding microorganisms (O'Neal et al. 2020).

It has been reported that the second messenger c-di-GMP mediates environmental signals perception resulting in changes in bacterial behaviour, including motility and biofilm formation (Jenal et al. 2017). Intracellular c-di-GMP levels inside cells were found to modulate chemotaxis in *A. brasilense* (O'Neal et al. 2020) and root internalization in *A. baldaniorum* (Sierra-Cacho et al. 2021).

Recently, deficiencies in the Hcp protein of the type VI secretion system (T6SS) have been shown to reduce the aggregation capacity of *A. argentinense* Az39, not only between *Azospirillum* cells but also between *Azospirillum* and the microalga *C. sorokiniana* (Cassán et al. 2021). Therefore, this secretion system might be involved in bacterial interaction and the formation of cell aggregates, a requisite for rhizosphere colonization.

Aggregate formation in *Azospirillum* can be affected by environmental conditions too. Blue light and white light increased aggregation in planktonic cultures of *A. argentinense* Az39 with respect to cells kept in the dark (Molina et al. 2021). Besides being involved in plant tissue colonization, aggregation may enhance *Azospirillum* survival under adverse conditions in the rhizosphere or in the water, one of the habitats this versatile bacterium may inhabit.

One of the best-known mechanisms through which bacterial cells communicate is quorum sensing (QS). This phenomenon regulates several processes in *Azospirillum* spp., like swimming and swarming motility, which are key for root colonization (Alexandre 2015). Although a full HDL-mediated QS system seems unusual in this genus, certain strains have been found to contain some QS-related genes (Vial et al. 2006). In bioassays with reporter strains, Gualpa et al. (2019) observed that *A. argentinense* Az39 could degrade natural and synthetic N-acyl homoserine lactones (AHLs) *in vitro* through quorum quenching, a mechanism consisting of QS suppression. Mechanisms of signal interception might then be prevalent in *Azospirillum* strains, regardless AHLs production. The fact that such mechanisms have emerged in this PGPR, particularly in *A. argentinense* Az39, points out the crucial role of these mechanisms in selecting an ecological niche, exchanging signals with the host plant (Tait et al. 2009), and adapting their lifestyles. As the molecular basis of *Azospirillum* interactions with the plant and the existing microbiota become clearer, more functions will be discovered.

Concluding remarks and perspectives

Although *Azospirillum* has been extensively used as an inoculant since the second green revolution, data on the actual prevalence of this PGPR in plants, soils, or seeds after inoculation are scarce. This contrasts with the great number of reports showing plant growth promotion by this bacterium under field conditions. The lack of precise methodologies to determine *Azospirillum* colonization under agronomic scenarios seems to be the main gap. Currently, most methods for monitoring *Azospirillum* strains used as inoculants are time-consuming, laborious, expensive, and unspecific

at the strain level. On the other hand, *Azospirillum* numbers in agronomic environments seem to be lower than those of other bacteria, hindering their molecular identification. Developing more reproducible, rapid, and inexpensive tools for tracking this versatile microorganism under field conditions is required to unravel *Azospirillum*-plant and *Azospirillum*-plant microbiome interactions. Omics approaches and advanced methodologies capable of identifying *Azospirillum* at the strain level, such as qPCR, FISH, or CRISPR, will allow progress in monitoring this ubiquitous bacterium widely used as biofertilizer in the context of sustainable agriculture.

Conflict of interest

I declare that there is no conflict of interest regarding the publication of this paper. I, the corresponding author, on behalf of all contributing authors, hereby declare that the information given in this disclosure is true and complete to the best of my knowledge and belief.

Author contributions

S. Nievas (Conceptualization, Data curation, Formal analysis, Writing – original draft), A. Coniglio (Conceptualization, Data curation, Formal analysis, Writing – original draft), W.Y. Takahashi (Conceptualization, Data curation, Formal analysis, Writing – original draft), G.A. López (Conceptualization, Data curation, Formal analysis, Writing – original draft), G. Larama (Data curation, Formal analysis), D. Torres (Conceptualization, Data curation, Formal analysis, Writing – original draft), S. Rosas (Conceptualization, Data curation, Formal analysis, Writing – original draft), R.M. Etto (Supervision, Visualization, Writing – review & editing), C.W. Galvão (Conceptualization, Data curation, Formal analysis, Writing – original draft), V. Mora (Conceptualization, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing), and F. Cassán (Conceptualization, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing)

Data availability

The data underlying this article are available in the article and in its online supplementary material.

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