REVIEW / SYNTHÈSE

Azospirillum-plant relationships: environmental and physiological advances (1990-1996)

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Abstract: This review presents a critical and comprehensive analysis of the developments in environmental and physiological studies related to *Azospirillum* interactions with plants based on information published between 1990 and 1996. It was designed as an update of a previous review with a similar scope. Apart from an update, this review emphasizes the central issues of *Azospirillum* research today, such as coinoculation with other microorganisms and hormonal studies, shows the less researched areas, and proposes possible avenues for the exploitation of this bacterium in areas other than agriculture.

Key words: *Azospirillum*, bacterial inoculation, plant-bacteria interaction, plant growth promoting rhizobacteria, rhizosphere bacteria.

Resume : La présente synthèse comporte une analyse critique et détaillée des connaissances issues des etudes physiologiques et environnementales reliées aux interactions de l'*Azospirillum* avec les plantes, d'après les informations publiées entre 1990 et 1996. Cette synthèse est une mise a jour d'une synthèse antérieure réalisée dans une optique similaire. Outre la mise a jour, les auteurs font ressortir les principales orientations de la recherche actuelle sur 1'*Azospirillum*, telles la co-inoculation avec d'autres microorganismes et les ètudes hormonales; ils signalent également les aspects les moins couverts et proposent des avenues possibles pour 1'exploitation de cette bactérie dans des domaines autres que 1'agriculture.

Mots clés : *Azospirillum*, inoculation bactérienne, interactions plantes-bactéries, rhizobactéries favorisant la croissance des plantes, bactéries des rhizosphères.
[Traduit par la rédaction]

Introduction

Since its rediscovery by J. Döbereiner and her collaborators in the 1970s, the species *Azospirillum* has gained the reputation of being the most studied plant-associative bacterium. Despite many successful experiments both in greenhouse and field, commercial application on a large scale has lagged. The main obstacle is the unpredictability and inconsistency of field results, especially when the grower has little time or knowledge to deal with bacterial inoculation (Okon and Labandera-Gonzalez 1994). This is discouraging to both the growers and the commercial industry. During recent years, several small-scale commercial inoculants have slowly entered the international market in Europe and South America.

Apart from direct agricultural application, *Azospirillum* is an excellent model for genetic studies of plant-associative bacteria in general. The largest portion of *Azospirillum* literature consists of genetic studies of almost all aspects of the bacterium and its association with plants. As the most researched associative bacterium, *Azospirillum* has become a cornerstone of rhizosphere research unrelated to its questionable field application. Applications other than those which are agriculturally related have arisen: production of poly-β-hydroxybutyrate (PHB) for medical use, degradation of pollutants, vitamin production, and purification of residual water are slowly being introduced into this field (for references see the respective sections).

Our last comprehensive review of the environmental and physiological aspects of *Azospirillum* interactions with plants was published in 1990 (Bashan and Levanony 1990) and will serve as a general past reference for this update. Commercial field applications, critical analysis of particular subfields, and genetic aspects of *Azospirillum* were the subjects of several recent reviews (Bashan 1993; Bashan et al. 1993b; Okon arid Itzigsohn 1995; Okon and Labandera-Gonzalez 1994; Vande Broek and Vanderleyden 1995), and therefore these issues will be discussed only briefly. The current review concentrates solely on results since 1990: earlier studies are cited only for enhancing clarity of the current review or giving a better perspective.

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A new species

Azospirillum irakense, the fifth *Azospirillum* species, was found in association with rice roots. This is the only species that has pectinolytic activities (Khammas and Kaiser 1991). The other species of this genus are *A. brasilense*, *A. lipoferum*, *A.* amazonense, and *A. halopraeferens*.

New host plants for isolation and for inoculation of the bacterium

A broad range of hosts is an obvious advantage for any given beneficial bacterium, eliminating the need for developing many specific crop-bacterial combinations that may confuse growers. In nature, a broad host range may help bacteria survive better. The full host range of *Azospirillum* has not been defined previously. Claims of *Azospirillum* specificity for certain cereal species are documented (see Bashan and Levanony 1990). The data published in recent years, however, show otherwise. *Azospirillum* strains had no preference for crop plants or weeds, or for annual or perennial plants, and can be successfully applied to plants that have no previous history of *Azospirillum* in their roots. It appears that *Azospirillum* is a general root colonizer and is not a plantspecific bacterium.

The claims that *Azospirillum* mainly enhances growth of cereal plants is inaccurate as well. Two *Azospirillum lipoferum* strains enhanced the growth of sunflower plants significantly (Fages and Arsac 1991) as did *Azospirillum brasilense* Cd for the development of oak seedlings (Zaady et al. 1993). Strains of *Azospirillum lipoferum* showed the strongest effect on the increase of nitrogen content and the mass and length of carrots (Govedarica et al. 1993, 1994) and on the yield of sugar beets in the field (Favilli et al. 1993). Rhizospheres of four tropical Nigerian plants (Delonix regia, Jecopina corsinea, Lagerstroemia indica, Lawsonia inermis) were favorable sites for *Azospirillum* (Russel and Ifiorah 1995). The response of mulberry to *Azospirillum brasilense* inoculation was beneficial at low levels of N fertilizer (Dayakar-Yadav and Nagendra-Kumar 1991). Inoculation of wild cardon cactus seeds with *Azospirillum brasilense* Sp-245 significantly enhanced seed germination and seedling growth variables related to survivability of the seedlings during the dry season (height, diameter, volume, and volume to surface ratio), but delayed spine senescence, a feature essential for plant defense against predation by rodents (Puente and Bashan 1993). All these new cases are in addition to the known plant growth promotion of tomato, eggplant, pepper, cotton (Bashan et al. 1989b), and mustard (Saha et al. 1985), and the ability of the bacterium to colonize at least 64 plant species, of which, 48 are weed species (Bashan and Holguin 1995).

Although reports about isolating *Azospirillum* from graminaceous plants are common, other reports showed that the bacterium is a natural inhabitant of many nongraminaceous plants. *Azospirillum* was detected in roots of coconut palms grown under diverse agronomic practices (George 1990) and within the stem nodules, root nodules, and stems of *Aeschynomene indica* and *Aeschynomene aspera* (Singh 1992). An *Azospirillum brasilense* like bacterium was isolated from the roots of five nongraminaceous crops (*Spinacia oleracea, Brassica chinensis, Brassica rapa* var. *pervidis, Glycine max, Cucumis sativus*) in Japan (Gamo and Ahn 1991).

Azospirillum has been previously isolated in various temperate zones (Bashan 1991*a*; De Coninck et al. 1988). Recently, it was isolated from 10 graminoid root species and adjacent soil collected from tundra and semidesert sites in the Canadian High Arctic (Nosko et al. 1994). Other curious isolations were from stems, leaf sheaths, and leaves (besides their roots) of several field-grown graminaceous plants like *Eleusine coracona* and *Setaria italica* (Agarwala-Dutt et al. 1991), and from the leaves of marine mangrove plants (Chaudhury and Sengupta 1991).

It appears that *Azospirillum* is a universal bacterium found almost everywhere.

Recent greenhouse field results

The first generation of *Azospirillum* inoculants is being slowly introduced into the agricultural market on a small scale (Fages 1992; footnote 3). The main factor preventing introduction of *Azospirillum* on a large scale is the unpredictability and the inconsistency of the results. These deficiencies have been known from the earliest days of *Azospirillum* application and have discouraged many commercial users. Twenty years of evaluation of data of field experiments showed 60-70% of all field experiments were successful with significant yield increases ranging from 5 to 30% . The main success factors were careful application of viable cells and proper maintenance of the experiments (Okon and Labandera-Gonzalez 1994; Okon and Itzigsohn 1995). Cells should be taken from the exponential phase of the culture (because these bacteria survive better in soil), rather than taking the inoculum from the stationary phase (Vandenhove et al. 1993). Although field inoculation is not the main area of *Azospirillum* research today (unless this has been done by commercial companies without publication of the results; Bashan et al. 1996), several recent greenhouse and field experiments, mainly in cereals, demonstrated once again its untapped potential.

In the greenhouse, the natural bacterial microflora from sunflower rhizosphere and strains of *Azospirillum lipoferum* were evaluated for their ability to promote sunflower growth in pots. Two strains of *Azospirillum lipoferum* strains and one of *Xanthomonas maltophilia* produced the best increased growth response (Fages and Arsac 1991). The response to *Azospirillum brasilense* Cd inoculation of chick-pea showed a significantly positive effect on the plant roots and shoots compared with uninoculated controls or to *Rhizobium* inoculated (but not nodulated) plants (Del Gallo and Fabbri 1990). The effect of inoculation with *Azospirillum brasilense* on growth and yield of *Sorghum bicolor* in hydroponic systems was a significant enhancement of dry matter content, leaf area development, and grain yield. At later stages of growth, leaf senescence was delayed in inoculated plants, thus favoring dry matter accumulation and grain filling (Sarig et al. 1990).

In field experiments in Argentina, corn inoculated with *Azospirillum lipoferum* showed double the seeds per ear, an increase in seed dry weight by 59 % , and a significant stimulation in root development at harvest time (Fulchieri and Frioni 1994).

[3] Okon 1994. Presentation at the 3rd International Workshop on Plant Growth-promoting rhizobacteria, Adelaide, Australia.

Significant effects on growth and yield of maize plants were obtained in one bacteria-plant combination and absent from another combination, indicating an unknown interaction (or perhaps a specificity?) between the plant genotype and bacterial strain (Garcia de Salomone and Döbereiner 1996). Similar phenomena were reported when local Nepalese wheat varieties were inoculated with local *Azospirillum* spp. (Bhattarai and Hess 1993) and a genotype-dependent response when mustard was inoculated with *Azospirillum* (Kesava Rao et al. 1990). Another corn study showed bacterial inoculation alone had no effect on green or dry matter yield. However, the use of nitrogen in combinations with *Azospirillum* produced significantly higher green and dry matter yields than those from inoculation or fertilization alone (Chela et al. 1993). Inoculation of Mediterranean herbaceous swards by *Azospirillum brasilense* Cd gave the best results in semiarid ecosystems, with aerial biomass production increasing about fourfold from inoculation. Zaady et al. (1994) based on the above results suggested that inoculation with *Azospirillum brasilense* on a commercial scale may offer means of increasing rangeland production without resorting to costly and ecologically unfavorable fertilizer application. *Azospirillum*-inoculated sugarcane seed pieces fertilized with a low level of nitrogen gave higher yields than those obtained from seed pieces inoculated, noninoculated, or fully fertilized. *Azospirillum*-inoculated seed pieces replaced about 60% of the required N fertilizer (Macalintal and Urgel 1992). Triticale plots fully fertilized and plots inoculated with *Azospirillum* and enriched with low rates of fertilizer of 120 kg N/ha gave a yield increase of about 53 % over noninoculated plants (Del Gallo et al. 1991). Inoculation of wheat with various strains of *Azospirillum* caused significant increases over controls in grain yield, ranging from 23 to 63 % (Caballero Mellado et al. 1992). Finally, inoculation of sunflower with *Azospirillum brasilense* Cd and *Azospirillum lipoferum* positively affected plant growth, especially under irrigation (Itzigsohn et al. 1995).

Though there is modest recent progress in greenhouse and field evaluation of inoculation, a major difficulty still persists in *Azospirillum* application. No study has directly addressed the major issues of inconsistency and unpredictability under farming practices. Only very carefully managed field experiments gave consistent results (Okon and Labandera-Gonzalez 1994). Without a proper practical solution (a farmer cannot grow an economical crop with the precision of a successful field experiment), it is unlikely *Azospirillum* will be in the field on a large scale soon.

Rhizocompetence and coinoculation with other microorganisms

As an outcome of the inconsistency in field inoculation of *Azospirillum*, a new research subfield has emerged, namely coinoculation of *Azospirillum* with other microorganisms. This promising new trend in the field of microbial inoculants represents the largest single topic of literature on *Azospirillum* in recent years. Coinoculation is based on mixed inoculants, combinations of microorganisms that interact' synergistically, or when *Azospirillum* is functioning as a "helper" bacterium to enhance the performance of other beneficial microorganisms. Studies done without plants indicate that some mixtures allow the bacteria to interact synergistically, by providing nutrients, removing some inhibitory products, or stimulating each other through physical or biochemical mechanisms.

Azospirillum can be associated with a wide variety of sugar- or polysaccharide-degrading bacteria. The coculture can be considered a metabolic association where the sugar-degrading bacteria produce degradation and fermentation products that can be used by *Azospirillum*. In a coculture of Bacillus and *Azospirillum*, pectin degradation by Bacillus and N_2 fixation by *Azospirillum* were enhanced (Khammas and Kaiser 1992). Coculture of *Azospirillum brasilense* and *Enterobacter cloacae* or *Azospirillum brasilense* and *Arthrobacter giacomelloi* led to a more efficient N_2 fixation than did either of the bacteria alone (Kaiser 1995; Lippi et al. 1992). When *Azospirillum* sp. DN64 was coinoculated with a mixture of cellulolytic fungi, its nitrogenase activity increased 22-fold (Halsall 1993).

Azospirillum has several characteristics which demonstrate its rhizocompetence (Bashan and Levanony 1990; Del Gallo and Fendrik 1994). One is its ability to accumulate large amounts of PHB in the presence of stress factors (see also the section Stress conditions). On nonwater-stressed soybean plants, *Azospirillum brasilense* Cd cells in the rhizosphere occurred as vibroid forms, whereas those on waterstressed plants were cyst-like. When water-stressed conditions were eliminated, the bacterial cells reverted to vibroid form with a concomitant increase in the bacterial population. Apparently, cyst-like formation is a natural response of the bacteria to water stress in the rhizosphere (Bashan et al. 1991*a*). Despite this, few studies have demonstrated the interaction between *Azospirillum* and native flora in soil. Considering that *Azospirillum* population is only a minute fraction of the microbial community in the rhizosphere, it is plausible to assume that many other microbial groups will affect its competence positively or negatively. Janzen and McGill (1995) demonstrated in physiochemically defined microenvironments that the proliferation of *Azospirillum brasilense* Cd depended on community-level interactions rather than on the genetically derived capability to fix N_2. About one third of 440 actinomycetes isolated from the soil and rhizosphere of a 60-year-old rye monoculture inhibited growth of *Azospirillum brasilense* and *Azospirillum lipoferum* (Kulinska and Kroczynska 1990).

Studies done with plants have shown that the beneficial effects which *Azospirillum* has on plants are enhanced when coinoculated with other microorganisms. Apparently, coinoculation allows the plants to have a more balanced nutrition and the absorption of N, P, and other mineral nutrients is improved.

Combined inoculation of *Azospirillum brasilense* and the phosphate-solubilizing bacteria Pseudomonas striata or *Bacillus polymyxa* on field-grown sorghum significantly increased grain and dry matter yields and N and P uptake as compared with single inoculation of individual organisms (Alagawadi and Gaur 1992). Pot experiments of barley showed a significant increase in grain yield by inoculation with mixtures of either *Azospirillum lipoferum* and the phosphate-solubilizing *Agrobacterium radiobacter* or *Azospirillum lipoferum* and *Arthrobacter mysorens*, compared with single inoculations. The N_2 fixation on roots and the accumulation of nitrogen in the plants was significantly higher when *Azospirillum*

lipoferum and *Agrobacterium radiobacter* were combined. The maximum positive effect of mixed inoculation on plant development was observed when the combined nitrogen in soil was in short supply. Field experiments with three barley cultivars confirmed the assertion that inoculation with these mixed cultures is superior to single culture inoculations (Belimov et al. 1995*a*, 1995*b*).

Dual inoculation of legumes with *Azospirillum* and *Rhizo*bium has been found to increase several plant-growth variables when compared with single inoculations. *Azospirillum* is considered a *Rhizobium* helper by stimulating nodulation, nodule function, and possibly plant metabolism (Andreeva et al. 1993). Phytohormones produced by *Azospirillum* promoted epidermal-cell differentiation in root hairs that increased the number of potential sites for rhizobial infection (Yahalom et al. 1990, 1991). As a result, more nodules were formed (Andreeva et al. 1993). In a lentil field experiment, mixed culture inoculation of *Azospirillum* and *Rhizobium signifi*cantly increased the total number of nodules, dry weight of nodules, and straw yield, and gave a considerable increase in grain yield (Yadav et al. 1992). Similar results were obtained in chick-pea (Del Gallo and Fabbri 1991; Fabbri and Del Gallo 1995). This interaction was further enhanced by organic matter present in the plant-growth medium (Del Gallo and Fabbri 1990).

Medicago sativa growing in pots under nonsterile conditions and inoculated with *Azospirillum* and *Rhizobium* showed a significantly greater shoot weight gain as compared with *Rhizobium* alone. Under gnotobiotic conditions, dual inoculation led to more nodules on the main root at intermediate *Rhizobium* concentrations (10^5 cfu/mL) and a greater root surface area at intermediate and high *Rhizobium* concentrations (10^5 and 10^7 cfu/mL, respectively). Pouch-grown seedlings, inoculated with *Rhizobium* alone, in combination with *Azospirillum*, or applied together with a flavonoid, luteolin (a nodulation gene inducer), showed that luteolin had effects similar to those of *Azospirillum* in increasing the main root nodule number and the total nodule number. Despite the increased main root nodule count in coinoculation, the same number of infection threads was formed, indicating that there was no improved regulation of nodule initiation with the combined treatment at the stage of infection thread formation (Itzigsohn et al. 1993).

Seven bacterial inoculants prepared with *Azospirillum brasilense, Azotobacter chroococcum, Klebsiella pneumoniae,* with or without *Rhizobium meliloti,* all improved alfalfa growth as measured by seven cuts per year over a 2-year experimental period. Combination with both Rhizobium and *Azospirillum* gave the highest increase (Hassouna et al. 1994). Similarly, combination of *Azotobacter* and *Azospirillum* on field-grown sugarcane was the best (Navale et al. 1995).

Cocultivation of *Azospirillum brasilense* Cd and Rhizo*bium leguminosarum* bv. *phaseoli* in culture medium resulted in coaggregation of the two bacteria and production of large flocs (see in the section Flocculation and cysts). Inoculation of beans with mixed flocs resulted in significant enhancement in nodulation and plant growth (Neyra et al. 1995). Contrarily, two studies report no enhancing effects on plants by combined inoculation with *Rhizobium* and *Azospirillum* as compared to single inoculations in peanuts (Raverkar and Konde 1990) and in the bean rajmash (Kundu et al. 1993). When *Azospirillum brasilense* was inoculated on Kallar grass seeds simultaneously with *Herbaspirillum seropedicae,* an endophyte root colonizer, the colonization of cells in roots by *Azospirillum brasilense* was inhibited (Stein et al. 1995).

Single or dual inoculation of wheat seedlings with *Azotobacter chroococcum, Azospirillum brasilense,* or *Streptomyces mutabilis* in sterilized soil resulted in significant stimulation of their populations in the rhizosphere. Single and dual inoculations also stimulated plant growth and significantly increased the concentrations of indoleacetic acid (IAA), P, Mg, N, and total soluble sugars in wheat shoots. Soil N content was increased by single inoculation with *Azotobacter* and by all dual inoculations (Elshanshoury 1995).

Inoculation of *Azospirillum brasilense* and *Azospirillum lipoferum* with the biocontrol agent fungus *Phialophora radicicola* significantly increased the shoot dry weight of wheat plants over inoculation by a single organism. A filtrate of the fungus exerted a stimulatory effect on the growth of both *Azospirillum* species, establishing a metabolic association between the microbes (Flouri et al. 1995).

Mixed inoculation of *Azospirillum* and the vesiculararbuscular mycorrhizae (VAM) fungus *Glomus intraradices* in sorghum created a synergistic interaction resulting in a significant increase in many plant-growth parameters including mycorrhizal infection with a concomitant increase in levels of root phosphatases (both alkaline and acid), an increase in the phosphorus content in plants, and enhanced uptake of nitrogen, zinc, copper, and iron. This double inoculation could replace the application of N and P fertilizers (Veeraswamy et al. 1992). Similarly, dual inoculation of wheat plants with *Azospirillum brasilense* and *Glomus* sp. or *Azospirillum brasilense* and an undefined VAM fungus, or dual inoculation of *Glomus macrocarpum* and *Azospirillum brasilense* in *Corchorus ollitorius* plants increased fresh and dry weights of shoots and roots (Al-Nahidh and Gomah 1991; Bali and Mukerji 1991; Gori and Favilli 1995). Inoculation of seeds of various genotypes of wheat with *Glomus fasciculatum* increased the grain and straw yields significantly, but a still higher increase in grain yield was recorded by coinoculation with *Azospirillum brasilense* (Singh et al. 1990). Spore germination of *Glomus fasciculatum* was stimulated by cell-free extracts of nonsymbiotic nitrogen fixers like *Azotobacter chroococcum, Beijerinck, Azospirillum brasilense* Cd, and *Azospirillum lipoferum* (Tilak and Dwivedi 1990). Mixed inoculation with VAM fungi and *Azospirillum brasilense* changed the morphology of strawberry roots (Bellone and de Bellone 1995).

In summary, coinoculation with *Azospirillum* and other microorganisms is one of the major frontiers of *Azospirillum* technology and perhaps the main area for future application.

New techniques for identification, location ire the roots, and the study of plant and bacterial interactions

Four approaches are common for monitoring the population and colonization sites of *Azospirillum* in roots and soil: the traditional methods, immunomethods, molecular methods, and combinations of these.

The traditional methods employ antibiotic resistance (Garcia de Salomone and Döbereiner 1996), direct microscopy and immunofluorescent techniques (Schloter et al. 1992), plate count methods (Puente and Bashan 1993), culture techniques, and routine morphological and physiological methods using commercial kits. These methods are popular, especially with laboratories preoccupied with monitoring of field experiments. They are concerned more with the fate of the inoculated bacteria than with the actual bacterial number or colonization sites. These methods usually define the bacterial genus, and sometimes the species levels, but have limitations at the strain level. Few can detect small amounts of bacteria (populations smaller than 10^4-10^5 bacteria/sample). Significant technical improvements using image processing of epifluorescence micrographs or confocal laser scanning microscopy have revived the immunofluorescence approach (Schloter et al. 1995).

Immunological detection methods have become increasingly important in microbial ecology for the tracking of specific microorganisms and for community analysis. For a reliable application of these techniques, the monoclonal or polyclonal antisera used have to fulfill several quality criteria. Cross-reactivity, cellular location of the antigenic determinant, affinity characteristics, and the expression of the antigenic determinant under environmental conditions have to be determined first (Schloter et al. 1994). Immunological methods can be used for the identification, quantification, and enrichment of specific bacteria in extracts and for the visualization of cells in situ. The sensitivity of advanced immunological methods can be compared with polymerase chain reaction (PCR) techniques (Schloter et al. 1995).

The most common and useful straightforward immunotechnique is the use of various enzyme-linked immunosorbent assay (ELISA) procedures (Levanony and Bashan 1991*b*). Their main drawback is that it is only possible to detect bacteria down to a density of 10^4 bacteria/mL of soil or root extract (Levanony et al. 1987). To overcome this limit and to avoid the high background readings of the enzymatic reaction of ELISA, incorporation of avidin-biotin complex into standard ELISA procedures significantly improved the quantitative detection and enumeration of *Azospirillum brasilense* (Levanony and Bashan 1990). To improve the detection level, a simple time-limited, liquid-enrichment procedure was developed, based on limited multiplication of *Azospirillum brasilense* in conventional semisolid medium and counting of the bacteria in the enriched medium by ELISA or on most probable number (MPN) techniques. The method can be used as a complementary procedure to ELISA and MPN techniques when low numbers of *Azospirillum brasilense* are present in the roots (Bashan et al. 1991*c*).

An easier, faster, and more sensitive method than the standard ELISA is immunoassay based on chemoluminescence. With this method, it was possible to quantify *Azospirillum brasilense* Sp-7 specifically down to a density of 100 bacteria/mL of soil extract (Schloter et al. 1992).

Molecular techniques, alone or in combination with immunotechniques, are at the forefront of detection techniques. They are mostly experimental, mainly used for precise detection at the laboratory research level, and as far as we know, have not entered the inoculation industry on a large scale (Fogher et al. 1995). However, from their proliferation and precision, one can predict in the future more detection techniques will be focused on molecular and on immunomolecular approaches.

Several methods of molecular fingerprinting of *Azospirillum* are useful in identifying species. Sometimes the methods are capable of identifying strains of the same species. Pulsed-field gel electrophoresis fingerprinting showed that azospirilla associated with different crops have a very similar genetic background (Eid and Sherwood 1995). Restriction fragment length polymorphism of the histidine operon, amplified 16S rDNA restriction analysis (Grifoni et al. 1995), probes of *Azospirillum* sp. based on the 16S rRNA (Kabir et al. 1995) and 23S rRNA genes (Kirchof and Hartmann 1992), plasmid analysis (Penot et al. 1992), and DNA restriction pattern analysis (Fani et al. 1993; Giovannetti et al. 1992) are easy, fast, reproducible, and reliable tools for identification of *Azospirillum* cells at the species level. When *Azospirillum brasilense* was marked by insertion of transposon Tn5 into its genome, the detection limit was as low as approximately 25 cells/g of dry soil (Christiansen-Weniger 1992*a*).

Reporter genes like *NifA-lacZ* were used to locate *Azospirillum brasilense* Sp-7 colonization of wheat roots (Katupitiya et al. 1995*a;* Vande Broek et al. 1993*).* Whole-cell hybridization with fluorescent-labeled, rRNA-targeted oligonucleotide probes was used for in situ monitoring of *Azospirillum brasilense* on wheat seedlings. Scanning confocal laser microscopy was used to overcome the disturbing effects of autofluorescence. This technique also allowed high-resolution analysis of the spatial distribution of bacteria in the rhizosphere (Assmus et al. 1995).

Apart from the above techniques, microcalorimetric output, the heat production of the culture, was used to measure bacterial survival in the soil and their metabolism in situ (Vandenhove et al. 1993).

The traditional techniques will survive for years to come for practical field monitoring, though it is obvious they will be eventually replaced by simpler versions or kits of the more advanced methods described above.

Attachment to roots and surfaces and sites of root colonization

The secure attachment of *Azospirillum* is essential for a long-term association with the host plant roots for three reasons. (*i*) If the bacteria are not attached to root epidermal cells, substances excreted by the bacteria diffuse into the rhizosphere and are consumed by nutritionally versatile microorganisms before reaching the plant. When the bacteria attach to the roots, part of these substances is diffused into the intercellular spaces of the root cortex. (*ii*) Without a secure attachment, water may wash the bacteria away from the rhizoplane to perish in the surrounding, nutrient-deficient soil. *Azospirillum* is known to survive poorly in many soils without host plants (Bashan and Levanony 1990*;* Bashan et al. 1995*a,* 1995*b*). *(iii)* Association sites on roots with no attached *Azospirillum* are vulnerable to other aggressive, possibly nonbeneficial colonizers.

Previous studies on *Azospirillum* attachment and root surface colonization revealed that the preferred sites for colonization, in most plant species studied, are the elongation and root hair zones where the bacteria form an aggregate type of colonization

supported by massive fibrilar material (for a review, consult Bashan and Levanony 1990). Recent data showed *Azospirillum brasilense* Sp-7 was restricted to the root hair zone. *Azospirillum brasilense* Sp-245 was found repeatedly at high density in the interior of root hair cells (Assmus et al. 1995). The colonization sites in some grasses corresponded to the areas where root mucigel was present. The area around the point of emergence of lateral roots usually shows high colonization (Bilal et al. 1993).

Binding kinetic data of *Azospirillum* to wheat seedlings indicated a saturated and specific number of available sites on the roots for the adhesion of *Azospirillum brasilense* Sp-245 (Zamudio and Bastarrachea 1994). Hydrophobic studies of *Azospirillum* cell walls showed that the bacterial cells are mildly hydrophobic, possibly involving hydrophobic proteins in the first stages of attachment of the bacterium to surfaces (Castellanos et al. 1996). The increase in cell surface hydrophobicity during growth was correlated with an increase of cell adhesiveness (Dufrene and Rouxhet 1966).

Azospirillum has two different phases of attachment to wheat roots. The primary adsorption phase is fast (reaches a maximum within 2 h of incubation), weak, and governed probably by bacterial proteins. The second phase (called anchoring) takes longer (it begins only after 8 h of incubation and reaches a maximum after 16 h), is stronger and irreversible, and probably is based on bacterial extracellular surface polysaccharides (Skvortsov et al. 1995; Del Gallo and Haegi 1990; Michiels et al. 1990; Zaady and Okon 1990). The adsorption and anchoring are probably different phenomena. This was demonstrated by the properties of two classes of *Azospirillum brasilense* attachment mutants. The first class of mutants, deficient in the production of a particular surface polysaccharide, has completely lost anchoring capability but maintains wild-type adsorption capacity (*Ads+ Anc-*). Mutants of the second class are defective in adsorption but not in anchoring (*Ads- Anc+*) (Michiels et al. 1990, 1991). The anchoring phase, characterized by the production of long fibrils and a large amount of mucigel-like substances, has been observed in roots of tomato, pepper, cotton, and soybean (Bashan et al. 1991*a*) and is probably a major factor in effective root colonization.

There are at least two different quantitative types of anchoring in this bacterium: a weak attachment to a nonbiological surface and a stronger attachment to roots. The anchoring of *Azospirillum brasilense* Cd to hydrophobic polystyrene was significantly less than to roots. Manganese-limited cells showed increased anchoring to roots (Bashan and Holguin 1993).

The polar flagellum of *Azospirillum brasilense*, which is primarily used for swimming, was involved in the attachment process of the bacteria to roots. The evidence presented was that (*i*) a nonmotile mutant has a drastically reduced capacity to adsorption to wheat roots, (*ii*) this bacterium lacked either polar or lateral flagella but was otherwise indistinguishable from the wild type, (*iii*) chemical or acid disintegration of the flagellum eliminated adsorption, (*iv*) Tn5 mutation produced three additional nonmotile mutants that were also deficient in adsorption, and (*v*) purified polar flagella bind to wheat roots in vitro (Croes et al. 1993).

It has been suggested that the mechanism of adhesion of *Azospirillum* to plant roots is mediated by lectins, such as wheat germ agglutinin, attached to the bacterial cell surface (Antonyuk et al. 1993; Karpati et al. 1995).

In summary, the attachment of *Azospirillum* is characterized by two-phase attachment, common also in marine bacteria, perhaps with the aid of the polar flagellum and affinity of the attachment to roots rather than to artificial surfaces. The bacterial attachment with aerial parts of the plants (described earlier in this review) remains unknown.

Stress conditions

Flocculation and cysts

Under various stress conditions, bacteria are capable of cyst and floc (macro, visible aggregates) formations, both of which improve survival. These phenomena can result from aging (Sadasivan and Neyra 1987), culture conditions (Bleakley et al. 1988), toxic metals (Gowri and Srivastava 1996), or water stress (Bashan et al. 1991a). Cystic cells rich in PHB survive better than those without PHB (Assmus et al. 1995; Oliveira and de Souza 1991; Zaady et al. 1993). The greatest growth-promotion effects on maize were observed when using *Azospirillum brasilense* cells containing 40% PHB (Fallik and Okon 1996). Cyst forms of *Azospirillum brasilense*, previously considered to be the dormant forms of the bacterium, were shown to be physiologically active forms in the wheat rhizosphere (Assmus et al. 1995). These cysts were capable of fixing nitrogen in the absence of an exogenous carbon source (Okon and Itzigsohn 1992). Under continuous culture and anaerobic conditions, cyst cells of *Azospirillum brasilense* Sp-7 and Sp-245, immobilized in alginate beads, showed high nitrate reductase activity (Ueckert et al. 1991).

By growing *Azospirillum* in the presence of fructose and nitrate, the synthesis of exocellular polysaccharides is promoted to induce the formation of cell aggregates (Arunakumari et al. 1992; Sadasivan and Neyra 1985, 1987). In contrast, malate-grown cells of *Azospirillum brasilense* Cd formed aggregates, whereas those grown with fructose remained separate. More fructose-grown cells were adsorbed onto corn roots and resulted in a significant increase in root surface area and root and foliage dry weight as compared with malate-grown cells. When inoculated onto oak seedlings, malate-grown cells significantly improved the development of the seedlings (Zaady et al. 1993), indicating that the preferred form of inoculation is an aggregate form. Conversely, a spontaneous mutant of *Azospirillum brasilense* Sp-7 did not flocculate and lacked the cell surface material of the parental strain, but had a superior N_2-fixation capacity when associated with wheat plants (Katupitiya et al. 1995a). It was also proposed that *Azospirillum brasilense* surface polysaccharides, which differ from the exopolysaccharides, are involved in the flocculation phenomenon (Michiels et al. 1990).

Because of the survival advantages of cysts of *Azospirillum* over vegetative cells, Neyra et al. (1995) suggested, and demonstrated on bean plants, the generation of inoculants composed of flocculants of *Azospirillum* and *Rhizobium*, which consist of a mixture of cysts and vegetative cells surrounded by a net of polysaccharides. As flocs can be produced readily on a large scale and separated easily from the growth medium with improved survival of the cells within the floc,

they may have potential in inoculant preparation. It is premature to predict the practical impact of the floc phenomenon.

Osmotolerance

The best performances of *Azospirillum* under field conditions are usually associated with nonoptimal conditions for plant growth (limited fertilization and water) and they occur mainly in semiarid agriculture. Semiarid agriculture is frequently associated with increased soil salinization (Matsumoto et al. 1994). Salt tolerance of a particular strain is of fundamental importance for the inoculation industry. Despite a few studies in the 1980s on salt tolerance of *Azospirillum*, and in particular the discovery of the halotolerant *Azospirillum halopraeferens* (Reinhold et al. 1987) and salt-tolerant *Azospirillum brasilense* strains (Rai 1991), little has been done recently on that issue.

A common cellular mechanism of osmotic-stress adaptation is the intracellular accumulation of organic solutes (osmolytes). All the osmolytes accumulating in several strains of *Azospirillum brasilense* during osmotic stress generated by NaCl were identified. Glycine betaine was accumulated in all strains, but proline and glutamate were accumulated only in *Azospirillum brasilense* SHS6, suggesting that the mechanism of osmotic adaptation resulted from enhanced uptake of osmolytes from the medium, biosynthetic increase of osmolytes, or both. The main osmolyte accumulate in *Azospirillum brasilense* SHS6 shifted from glutamate to proline as the osmotic strength of the medium increased (Madkour et al. 1990). A new osmolyte, a linear β(1,3),β(1,6)-glucan was detected in the periplasm of *Azospirillum brasilense* cells growing in a medium of low osmotic strength (Altabe et al. 1994). *Azospirillum brasilense* is able to use glycine betaine as a powerful osmoprotectant. The uptake of this compound is strongly stimulated by salt stress but significantly reduced by cold osmotic shock. A glycine betaine binding protein in periplasmic shock fluid obtained from high osmolarity grown cells was detected (Riou and Le Rudulier 1990; Rion et al. 1991).

It can be concluded that in the genus *Azospirillum*, tolerance towards high concentrations of NaCl, sucrose, or polyethylene glycol increases in the order *A. amazonense, A. lipoferum, A brasilense,* and *A. halopraeferens* (Hartmann et al. 1991).

Apparently, *Azospirillum* inoculation improved plant growth under water-stress conditions as was demonstrated also in the 1980s. Subjecting inoculated Sorghum bicolor plants to an osmotic stress in hydroponic systems diminished the adverse effects caused by osmotic stress, such as reduction of leaf senescence (Sarig et a. 1990). Coleoptile height and fresh and dry weight of inoculated wheat seedlings with *Azospirillum brasilense* Sp-245 were enhanced despite the water stress (Alvarez et al. 1996).

Effect of pesticides

It is clear that *Azospirillum* has been used in common agricultural practices and is not, and will not be, restricted to organic farming. Under such circumstances, it will encounter all the pesticides used in intensive agriculture. Agricultural pesticides commonly have side effects on nontarget microorganisms. Surprisingly, this important issue attracted a negligible amount of research in the past and not much more in recent years. The majority has been done under in vitro conditions.

Herbicides

The common herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is used in cereals and grasses to control broadleaf weeds. At a very low concentration of 1 mM, 2,4-D inhibited *Azospirillum* cell growth, an effect that was reversed either by transferring bacteria to a control (2,4-D-free) medium or to a 2,4-D-treated medium supplemented with polyamines. In vitro protein synthesis (Rivarola et al. 1992), and also N_2 fixation and growth (Martinez-Toledo et al. 1990), in a pure culture of *Azospirillum brasilense* were inhibited.

Another commonly used herbicide, Thiobencarb, was studied on two species of *Azospirillum* in pure cultures and in association with rice under laboratory and field conditions. Thiobencarb had no effect on *Azospirillum lipoferum* 4B, whereas it inhibited the growth of *Azospirillum brasilense* N040. Although Thiobencarb had a negative effect on the growth of aseptically grown rice plantlets, when they were first inoculated with *Azospirillum* strains in a gnotobiotic condition containing the herbicide, the N_2 fixation was similar to the control lacking the herbicide (Omar et al. 1992). Low levels of Thiobencarb applied to three tropical rice soils incubated under nonflooded conditions inhibited the *Azospirillum* population (Jena et al. 1990).

Azospirillum brasilense grown in a chemically defined medium and a dialyzed soil medium in the presence of Alachlor and Metolachlor had lower N_2 fixation and lower levels of ATP compared with nontreated control cells. The adverse effects due to Metolachlor disappeared after 48 h, indicating that *Azospirillum brasilense* can tolerate high concentrations of that herbicide (Salmeron et al. 1991). The sulfonylurea herbicides (Chlorosulfuron and Rimsulfuron), however, inhibited the growth of *Azospirillum*. Surfactants in commercial formulations significantly enhanced Rimsulfuron toxicity to *Azospirillum* (Forlani et al. 1995).

Insecticides

The organochlorine Thiodan inhibited growth and N_2 fixation of *Azospirillum lipoferum*. The active ingredient endosulfan was nonspecifically bound to proteins and mainly adsorbed on the cell envelope (Buff et al. 1992).

Azospirillum brasilense can tolerate high concentrations of diflubenzuron, which significantly increased growth, N_2 fixation, and amounts of ATP in the cells (Sánchez et al. 1994). Carbofuram, a common insecticide in rice cultivation, significantly stimulated N_2 fixation in *Azospirillum* so. (Jena et al. 1992).

Concentrated emulsions of commercial formulations of six insecticides were added to *Azospirillum lipoferum* medium. Among the tested products, only Bidrin did not inhibit growth and N_2 fixation. Motility and cell volume were reduced with Kelthane, Thiodan, Lorsban, Folidol, and Sevin. The relatively high inhibitory concentrations suggest that *Azospirillum* cells have some protective mechanism against these insecticides (Langenbach et al. 1991).

Fungicides

The fungicides Captan and Thiram were very toxic to *Azospirillum brasilense.* Cell growth and N_2 fixation were markedly inhibited

by low concentrations of the two fungicides (Gallori et al. 1991).

Bacteriocides

Surprisingly, the effect of bacteriocides, especially the commonly used copper formulations, have not been reported with regard to *Azospirillum*.

Although in vitro studies may indicate a potential deleterious effect of a pesticide on *Azospirillum*, they give little information about the actual effect of the compounds in more realistic farming practices. As such, the current knowledge regarding the effect of pesticides on *Azospirillum* is below the threshold for meaningful conclusions.

Motility in vitro and in situ

The common inoculation technology usually involves standard agrotechnical sowing devices that are inaccurate from the microbial standpoint; i.e., they are unable to ensure that the bacteria will encounter the emerging root. Bacterial movement from the inoculation site to the root site is essential if root colonization is to occur. This distance can range from a few micrometres to several centimetres. *Azospirillum* cells do not disperse with percolating water but are absorbed onto soil particles. Furthermore, passive possible dispersion by water in semiarid conditions lacking sufficient water where *Azospirillum* performed best (Caceres et al. 1996) cannot explain how the entire root system is colonized. In plant-free, water-saturated soils, *Azospirillum* stayed at the inoculation site and did not move (Bashan and Holguin 1995). It is reasonable to assume that another efficient bacterial dispersion mechanism exists, i.e., in situ chemotaxis. However, most work on motility of *Azospirillum* has been done on in vitro chemotaxis (Zhulin and Armitage 1992).

In vitro chemotaxis and aerotaxis

Zhulin and Armitage (1993) showed that the polar flagella of free-swimming *Azospirillum brasilense* cells can rotate both clockwise and counterclockwise. No methylation-dependent pathway for chemotaxis in *Azospirillum brasilense* was found. As *Azospirillum brasilense* lacks dedicated chemoreceptors or methyl-accepting chemotaxis proteins, the range of chemicals to which *Azospirillum brasilense* shows chemotaxis and the lack of true repellents all indicated a chemotaxis mechanism other than the common one for *Escherichia coli*. Recently, it was shown that the main chemotaxis pathway might be dependent on proton-motive force sensing that powered the bacterial flagella motor. Changes in chemotactile behavior of *Azospirillum brasilense* Sp-7 coincided with changes in membrane potential, the main component of proton-motive force. This may indicate that *Azospirillum brasilense* responds chemotactically to intracellular metabolic changes and the intracellular energy status of the cell and not to external signal molecules (Zhulin and Taylor 1995; Zhulin et al. 1995).

Azospirillum lipoferum and *Azospirillum brasilense* respond chemotactically to threshold concentrations (in the nanomolar to picomolar range, concentrations relevant to the soil and rhizosphere environments) of aromatic compounds. *Azospirillum* clearly has more sensitive chemosensory mechanisms than reported for several other soil bacteria (Chen et al. 1993; López de Victoria and Lovell 1993; López de Victoria et al. 1994).

In the last two decades, *Azospirillum* chemotaxis was evaluated for many naturally occurring root exudates (see in Bashan and Levanony 1990). In addition to those, *Azospirillum brasilense* responds chemotactically to tobacco root exudates (Maheswari and Purushothaman 1990). When 15 *Azospirillum lipoferum* and *Azospirillum brasilense* strains, originating from different C_3 and C_4 crop plant roots, were compared for their chemotactile response to sterile wheat and maize root exudates, the results suggested a general chemotactile behavior by azospirilla, rather than a specific host-dependent response (Fedi et al. 1992). Chemotaxis is an indispensable behavior for successful root colonization. When a mutant defective in chemotaxis but otherwise motile was challenged with a chemotactile wild-type strain, the latter outcompeted the mutant for Kallar grass roots (Kimmel et al. 1990) .

Aerotaxis and microaerophylic growth are the fundamental characters by which *Azospirillum* was rediscovered (Döbereiner and Day 1976) and is routinely isolated today (Bashan et al. 1993*a*). Several studies on aerotaxis were published in the 1980s (e.g., Reiner and Okon 1986) showing the significance of this mechanism. In the 1990s, only one study has addressed this issue. Formation of a microaerophylic band (pellicle) in the zone of optimal oxygen concentration was similar to the one formed in a redox gradient of tetramethyl *p*-phenylenediamine where oxygen uptake was inhibited by cyanide. It is suggested that electron flow through the redox chain rather than binding oxygen to terminal oxidases may be a signal in the aerotactic response of *Azospirillum brasilense* (Grishanin et al. 1991).

In situ chemotaxis

Despite the obvious application of in vitro chemotaxis research to plants growing in soils, only a handful of studies have addressed this subject. It is known that *Azospirillum brasilense* can move in situ towards plants in soils for relatively large distances (in centimetres) (Bashan 1986*a*; Bashan and Levanony 1987; Bashan et al. 1994*a*). When the root-to-root movement of *Azospirillum brasilense* in sand and soil trays was measured using a motility-deficient strain of the wild-type motile strain Cd, the motile strain moved from the inoculated wheat and soybean roots to noninoculated roots. There, upon arrival they formed a band-type colonization composed of bacterial aggregates encircling a limited part of the root. The nonmotile strain did not move and stayed at the inoculation site and root tips. Attractants and repellents were the primary factors governing this motility (Bashan and Holguin 1994; Bashan et al. 1994*b*). Only the wild-type motile strain moved between different weeds regardless of the species, botanical family, or whether they were annual or perennials (Bashan and Holguin 1995). Apparently, the interroot movement of *Azospirillum brasilense* is an essential preliminary step in the root-bacterium recognition system. Bacterial motility in situ might have a general role in getting *Azospirillum* cells to the site where later firmer attachment favors root colonization. In summary, *Azospirillum* travels towards plants is a nonspecific active process, not directly dependent on nutrient deficiency but a consequence of a nonspecific bacterial chemotaxis influenced by the balance between attractants and possible repellents leaked by the roots.

Bacterial movement to noninoculated roots from inoculated roots that were still exuding nutrients can be explained in at least two ways. (*i*) The bacterial population on the roots consumed more nutrients than the root could supply. This created a temporary nutrient-deficient microenvironmental gradient which may have stimulated migration towards alternative nutrient sources. (*ii*) *Azospirillum* cells exhibited mainly an aggregate type of colonization, which restricted cell movement because of the production of extensive fibrillar materials. These aggregates provide an ecological advantage over any single bacterial cell in the competition for nutrients in the rhizoplane. Therefore, one may propose that to survive, single cells must locate sites that lack aggregate colonization, so they migrate to and colonize noninoculated roots. Moving to an uncolonized root may be a way in which a single bacterium can effectively compete with bacterial aggregates for nutrients.

Proposals for mode of action

The mode of action for *Azospirillum* has not been defined or agreed upon despite 20 years of research, although the bacterial physiological properties are well defined (Hartmann and Zimmer 1994). Almost all the old proposals are still valid (see in Bashan and Levanony 1990) and among them, some have accumulated supportive data during recent years. These are hormonal activities, N_2 fixation, undefined signal molecules that interfere with plant metabolism, nitrite production, and improved root growth in general.

Hormones

Azospirillum can produce in vitro the phytohormones IAA, gibberellins, cytokinins (losipenko and Ignatov 1995; Patten and Glick 1996; Rademacher 1994; for earlier literature, see Bashan and Levanony 1990), and ethylene (Strzelczyk et al. 1994). Sometimes, external application of synthetic hormones or hormones purified from bacterial culture imitated the positive effects of *Azospirillum* on root development and morphology. Examples of these activities are presented below.

Gibberellin A_3 production by stationary-phase, pure cultures of *Azospirillum lipoferum* appeared to be correlated with culture viability and polysaccharide excretion, favored in turn by a specific N concentration and initial pH of the culture (Piccoli and Bottini 1994). Gibberellin GA_3 had effects similar to *Azospirillum lipoferum* inoculation in increasing root hair density. Inoculation of corn seedlings with *Azospirillum lipoferum* changed the gibberellin status of the seedlings (Fulchieri et al. 1993). Janzen et al. (1992) found that coculture of *Azospirillum brasilense* and the barley straw degrading fungus *Trichoderma harzianum* produced a substantial increase of GA. The amounts of gibberellins and cytokinins produced by the mixed culture of *Azospirillum brasilense* and Arthrobacter giacomelloi were higher than those of the single cultures (Cacciari et al. 1989), suggesting that the interactions between the rhizosphere inhabitants may affect their secondary metabolism and indirectly the plants (Cacciari et al. 1989).

Three distinct pathways for IAA biosynthesis in *Azospirillum* have been described. One does not use tryptophan as a precursor (Costacurta and Vanderleyden 1995; Vande Broek and Vanderleyden 1995). Screening the tryptophan-dependent IAA production of different *Azospirillum* species revealed that *Azospirillum irakense* KA3 released 10 times less IAA into the medium than *Azospirillum brasilense* Sp7 (Zimmer et al. 1991), and that oxygen is required during the conversion of tryptophan to IAA (Bar and Okon 1995). The extent of use by soil bacteria of exogenic L-tryptophan for IAA biosynthesis in the root exudates was found to be 0.28 -1.0
(Kravchenko et al. 1994). In liquid culture of *Azospirillum brasilense* Cd, the concentration of IAA increased rapidly with the beginning of the stationary phase. The sole carbon source in the substrate (Dr.-malic acid) was identified as the limiting growth factor. This suggested that the high increase in IAA production in the stationary phase is the expression of an overall change in cell metabolism when the carbon source is exhausted (Omay et al. 1993). Production of major quantities of IAA and gibberellins at relatively older stages of the bacterial growth may imply that this might be relevant to the field rhizosphere interaction of *Azospirillum* with plants where the bacteria are seldom at the logarithmic phase, yet affect plant development. This may also explain why very old aggregated cultures (flocs) induced positive plant response after inoculation (Neyra et al. 1995).

Many studies have suggested the involvement of auxin produced by *Azospirillum* in root morphology (Baca et al. 1994; for recent reviews, see Costacurta and Vanderleyden 1995; Patten and Glick 1996). Inoculation of *Azospirillum brasilense* Cd on *Arabidopsis thaliana* increased the length of individual root hairs at least twofold (Dubrovsky et al. 1994). When inoculated onto wheat seedlings *Azospirillum brasilense* SpM7918, a very low IAA producer, showed a reduced ability in promoting root system development and decreased the potential of the plants' capacity for mineral uptake compared with plants inoculated with the wild type (Barbieri et al. 1991; Barbieri and Galli 1993). In contrast, sorghum roots inoculated with *Azospirillum brasilense* Cd showed a lower level of free IAA than noninoculated roots, while root morphology was extensively modified (Barbieri et al. 1995). Similarly, germinated wheat seedlings grown in a hydroponic system showed that incubation with *Azospirillum* drastically enhanced the formation of lateral roots, whereas IAA did not. External addition of IAA increased the dry weight only slightly, similar to *Azospirillum* (Bothe et al. 1992).

Cell-free extracts and culture supernatants prepared from *Azospirillum brasilense* Cd and the cytokinin benzyladenine (BA) significantly increased the number of nodules of burr medic seedlings grown in pouches when applied with *Rhizobium* inoculation, as compared with inoculation with *Rhizobium* alone (Yahalom et al. 1990). Exogenous IAA or BA stimulated or inhibited root elognation of burr medic seedlings depending on the concentration used (Yahalom et al. 1991). Cytokinin BA reduced nodulation significantly relative to alfalfa plants inoculated only with *Rhizobium*. These results clearly show that the influence exerted by *Azospirillum* on plants is not due solely to phytohormones excretion (Itzigsohn et al. 1993).

It is obvious that phytohormones, especially IAA secreted by *Azospirillum*, play an essential role in plant growth stimulation in general, and in stimulating symbiosis between legumes and rhizobia. However, to attribute a phenomenon of nonspecific growth promotion of numerous plant species resulting from *Azospirillum* inoculation to one substance is oversimplistic, albeit useful as a research tool for understanding the mechanisms of action

of the bacteria (Itzigsohn et al. 1993). For a better evaluation of the role of hormones in general and IAA in particular in plant promotion, there is a need for a mutant totally deficient in capacity IAA production. This mutant is unavailable as yet (Vande Broek and Vanderleyden 1995). The same is true for all the other phytohormones. Finally, to be able to state that hormonal effects are the main mechanism in plant growth promotion by *Azospirillum*, other, yet unperformed, studies on nitrite produced by *Azospirillum* must be done, since nitrite provokes an equally drastic increase in lateral root formation on wheat (Bothe et al. 1992). Similarly, other proposed mechanisms (see below) must be demonstrated to be of only minor importance.

Nitrogen fixation

Nitrogen fixation was the first mechanism proposed to explain improved plant growth following inoculation with *Azospirillum*. This was mainly because of an increase in the number of nitrogenous compounds and the nitrogenase activity in inoculated plants. Several years later, however, studies showed that the contribution of N_2 fixation by *Azospirillum* to the plant is minimal and ranged from 5 to 18 % of the total plant increase. Regularly, the contribution is smaller than 5 % . *Nif*- mutants were capable of increasing plant growth similar to that by the wild-type N_2 fixer (Bashan et al. 1989c). These findings almost caused an abandonment of the N_2 -fixation aspects of *Azospirillum* except for pure genetic studies (for a review, see Bashan and Levanony 1990). Recently, the interest in N_2 fixation in environmental and physiological studies of *Azospirillum* has arisen.

Azospirillum brasilense Sp-7 did not synthesize nitrogenase at 42°C, nor was the enzyme stable at this temperature. But, in *Azospirillum brasilense* Sp-9, nitrogenase activity was stable and showed the highest acetylene reduction activity at 42°C (Aggarwal and Chaudhary 1995). The nitrogenase activity of *Azospirillum* has been found to increase when grown in mixed cultures with other bacteria, even if they come from completely different habitats (Holguin and Bashan 1996; Khammas and Kaiser 1992; Lippi et al. 1992). Apparently, some mixed cultures provide conditions more suitable for N_2 fixation than those present in pure cultures. An example for an extremely unlikely association is the mixed culture of *Azospirillum brasilense* Cd and the non-N_2-fixing, marine mangrove rhizosphere bacterium *Staphylococcus* sp. that increased the N_2 fixation of the former. The effect was stronger when diluted Staphylococcus supernatant was added to *Azospirillum brasilense* culture and was partially due to release of aspartic acid from the Staphylococcus sp. cells (Holguin and Bashan 1996). In other studies, N_2 fixation of *Azospirillum brasilense* Sp-245 was enhanced by the addition of wheat germ agglutinin (Antonyuk et al. 1993, 1995; Ignatov et al. 1995).

Because *Azospirillum* does not secrete significant amounts of ammonium and provides the plant with minimal amounts of nitrogen, spontaneous mutants of *Azospirillum brasilense* were selected that excrete substantial amounts of NH_4^+. To counteract the ecological disadvantage this represents, the bacteria were established inside the roots through the induction of para-nodules (root structures that externally resemble legume nodules and can be induced by adding 2,4-D to roots) (Christiansen-Weniger 1992*a*, 1992*b*; Christiansen-Weniger and Vanderleyden 1994; Tchan et al. 1991). When plants were grown on a N-free medium, these mutants were responsible for significant increases in root and shoot dry matter and total plant nitrogen compared with wild type treated or noninoculated plants. Analysis of $^{15}N_2$ in these plants showed that these mutants were able to transfer more nitrogen to the host plants than the wild-type strain (Christiansen-Weniger and van Veen 1991). These results show graminaceous plants are potentially capable of establishing an association with diazotrophic bacteria in which the ammonium-excreting bacteria provide the host plants with a source of nitrogen. Other para-nodule studies of wheat seedling roots (Katupitiya et al. 1995b; Sriskandarajah et al. 1993) showed that the nitrogenase activity in para-nodules was higher than in inoculated roots without para-nodules (Tchan et al. 1991; Yu et al. 1993; Zeman et al. 1992). Para-nodule results add a new dimension to research on biological N_2 fixation, even if extensive developmental and biochemical modification of the para-nodule system is required before effective N_2 fixation can be achieved (Christiansen-Weniger 1994; Kennedy 1994; Kennedy and Tchan 1992). In contrast, the use of $^{15}N_2$ in pot hydroponic systems provided clear evidence for the absence of biological N_2 fixation in Sorghum bicolor inoculated with *Azospirillum* (Sarig et al. 1990).

An innovative strategy in the study of the *Azospirillum*-plant interaction is through tissue and root cultures (de Freitas and Germida 1990). Callus culture of the grass *Eulaliopsis binata and* of sugar cane *Saccharum offacinarum* were cocultivated with *Azospirillum* and showed positive nitrogenase activity after 12 months of inoculation (Gosal et al. 1990).

Apparently, the dismissal of N_2 fixation as a mechanism for plant growth promotion by *Azospirillum* was premature.

Signal molecules

Whatever the exact mechanism, the fact that *Azospirillum* affects plant cell metabolism from outside the cell (without entering the intact plant cells) suggests that the bacteria are capable of excreting and transmitting a signal(s) which crosses the plant cell wall and is recognized by the plant membranes. This interaction initiates a chain of events resulting in the observed altered metabolism of the inoculated plant. Since plant membranes are extremely sensitive to any change, their response may serve as a precise indicator for *Azospirillum* activity at the cellular level.

Short exposure of wheat roots to live *Azospirillum brasilense* Cd significantly enhanced the proton efflux of the root (essential for plant well being and associated with many metabolic root functions) 5 h after inoculation. Bacteria in the logarithmic phase are required for this enhancement, which is of a triggering nature (Bashan 1990; Bashan et al. 1989*a*). Additionally, inoculation of soybean seedlings with *Azospirillum brasilense* Cd significantly reduced the membrane potential in every root part and this reduction was greatest in the root elongation zone (Bashan 1991*b*; Bashan and Levanony 1991). Inoculation of soybeans and cowpea with *Azospirillum brasilense* Cd increased proton efflux from their roots and changed the phospholipid content in cowpea plant membranes (Bashan et al. 1992). Recently, it was suggested that motility of *Azospirillum* also is driven by protonmotive force of bacterial origin (Zhulin et al. 1995). Although the nature of the

released signal molecule is as yet unknown, it is proposed that plant membranes are probably a primary target for *Azospirillum* on plant roots.

It is also possible that a receptor in *Azospirillum brasilense* is involved in the binding of wheat germ agglutinin (WGA; one of the most studied plant lectins) (Antonyuk et al. 1993). This binding induced changes in the cellular metabolism of *Azospirillum brasilense* Sp-245 and promoted nitrogen fixation, the excretion of ammonium ions, and the synthesis of IAA (Antonyuk et al. 1993, 1995). WGA changed the relative proportion of acidic phospholipids of the membrane. It is possible that acidic phospholipids participate in transmembrane communication. It was suggested that WGA may function as a signal molecule in the *Azospirillum* -plant association (Antonyuk et al. 1995).

Nitrite production

Nitrite, either added directly or excreted by *Azospirillum* in nitrate respiration, similarly causes a sharp increase in the formation of lateral roots. It is possible that the growth promotion effect on wheat roots by *Azospirillum* is because of formation of nitrite (Bothe et al. 1992). This avenue has not been pursued in recent years.

General improvement of plant growth

Improved root growth and function were proposed in the late 1970s as a possible mechanism by which *Azospirillum* affects plant growth (Fallik et al. 1994). In hydroponic systems under greenhouse conditions, inoculation with *Azospirillum brasilense* increased the total number and length of adventitious roots of Sorghum bicolor 33-40% over noninoculated controls. This resulted from a higher rate of growth, earlier root appearance, and a greater elongation rate of individual sorghum roots (Sarig et al. 1992). This avenue has not been pursued in recent years.

Enhanced mineral uptake

Enhanced mineral uptake in the plant as a result of *Azospirillum* inoculation was a popular explanation for the inoculation effects in the 1980s (for a review see Bashan and Levanony 1990). Although some studies showed accumulation of nitrogen and other minerals in the inoculated plants, others showed that enhanced growth of wheat and soybeans was not necessarily because of a general enhancement of mineral uptake (Bashan et al. 1990). This avenue has modestly been pursued in recent years (Hernandez and Sarmiento 1995; Stancheva et al. 1995).

Additive hypothesis

The modes of action of *Azospirillum* proposed over the last two decades point to the possibility that perhaps there is no major single mechanism involved. Bashan and Levanony (1990) proposed the possibility of more than one mechanism involved at the same time, with individual mechanisms being less significant when evaluated separated. For example, N_2 fixation contributes less than 5 % of the observed effect of *Azospirillum* on the plant. As such, it does not fully explain yield increases. When combined with the effect of other small mechanisms, this may be a significant contribution. The combined activities of all the involved mechanisms may be responsible for the large measured effects of *Azospirillum* spp. inoculation on plants. With the exact mechanism unknown, it would be more practical to look at the effect of *Azospirillum* spp. on the whole plant rather than only at organ, tissue, cellular, or subcellular levels. A literature analysis of most of the known cases of the effect of inoculation on the root/shoot (S/R) ratio shows that the general effect of *Azospirillum* spp. on the entire plant was largely overlooked. From the changes the bacteria produce in the S/R ratio, it appears that it also participates in the partitioning of carbon compounds within the plant, a phenomenon well recognized as multiparametric (Wilson 1988). This analysis provides experimental data (although collected from many diverse studies) indicating that the mode of action of *Azospirillum* spp. is probably composed of multiple mechanisms (Bashan and Dubrovsky 1996), as previously suggested (Bashan and Levanony 1990).

Soil persistence and survival

When *Azospirillum* is not inoculated onto the seeds, it is usually inoculated directly into the soil. There, once the bacteria are released from the inoculant carrier, they are subjected to all the chemical and physical soil factors in addition to competition from the indigenous soil microflora. In the past, conflicting evidence was presented about soil persistence and survival of the bacteria. In Brazil, this rhizosphere bacterium is also capable of unlimited persistence in soils, whereas in Israel, and in some American and Canadian soils, it survived poorly (for review, see Bashan and Levanony 1990*).* *Azospirillum brasilense* Cd was well absorbed on light- and heavy-textured soils but poorly absorbed on quartz particles (Levanony and Bashan 1991*a).* Inoculation and incubation of *Azospirillum brasilense* Cd in pure quartz sand resulted in cell attachment to sand particles by a network of fibrillar material that is essential for anchoring. Inoculation of the sand with an aggregate-deficient mutant resulted in no detectable fibrillar formation. Rinsing of the sand desorbed the cells entirely. Protease treatment of colonized sand significantly decreased attachment. The survival period of wild-type cells was longer than that of the deficient mutant (Bashan et al. 1991*b).* The survival of *Azospirillum brasilense* Cd and Sp-245 was evaluated in 23 plant-free soils of 23 soil types obtained from a wide range of environmental conditions and also in the rhizosphere soil of plants growing in the same soil types. The survival rate was analyzed for 15 common soil variables. The bacteria survived well in all the rhizospheres tested, regardless of soil type, bacterial strain, or origin of the soil. In the absence of plants, the general survival characteristics were related mainly to the geographic origin of the soil and not to prevailing environmental conditions; i.e., in some soils they survived well and in others survival was poor. The only direct effect of soil variables on survival was that the concentrations of $CaC0_3$ and sand both have adverse effects (Bashan et al. 1995*a*, 1995*b).*

Since almost every soil is different from a microbial standpoint, additional comparative studies (like Bashan et al. 1995*a*,, 1995*b)* are still needed to gain a meaningful conclusion about the general survival capacity of the bacterium.

Airborne transmission

When *Azospirillum* is inoculated into the plant rhizosphere soil, many experimental designs take into consideration the possibility

of contamination of the controlled plants by bacterial transfer with excess irrigation water. Random sampling of noninoculated plants revealed that some plants contained the inoculated strain in their roots despite all precautions. The source of this contamination remains unknown. In controlled environments, plants inoculated with *Azospirillum brasilense caused* the contamination of noninoculated plants via air transmission. In a temperate agricultural zone (Ohio, U.S.A.), the bacteria were found in the air year-round. Apparently, contamination from *Azospirillum*-inoculated plants may occur via airborne bacteria. The existence of an airborne phase in a rhizosphere bacterium like *Azospirillum* presents a risk of uncontrolled airborne contamination from inoculated rhizobacteria (Bashan 1991*a*) and should not be ignored.

Azospirillum as a biocontrol agent

Azospirillum is not yet known as a biocontrol agent of soilborne plant pathogens. However, some evidence shows that this activity has been overlooked. *Azospirillum lipoferum* M produced catechol-type siderophores under iron-starved conditions that exhibited antimicrobial activity against various bacterial and fungal isolates (Shah et al. 1992). Twenty seven *Azospirillum* isolates produced bacteriocins that inhibited the growth of several indicator bacteria (Tapia-Hernandez et al. 1990). The effect of *Azospirillum brasilense on* crown gall formation in dicotyledonous plants was studied after inoculating them with virulent strains of *Agrobacterium tumefaciens.* When the wounded tissues of grapevines and carrot disks were preinoculated with the live cells of Azo*spirillum brasilense* 94-3 or Sp-7, the development of the typical bacterial galls was inhibited and the protective effect of *Azospirillum* lasted over a 24-h period (Bakanchikova et al. 1993). When *Azospirillum brasilense* Cd was mixed in a culture with the mangrove rhizosphere bacterium *Staphy*loccus sp., the population of the latter was significantly reduced (Holguin and Bashan 1996). Three strains of *Azospirillum brasilense* and *Azospirillum halopraeferens* failed to induce the defense mechanisms of cowpea calli as did its pathogen *Pseudomonas syringae* (Alcaraz-Melendez et al. 1996). Furthermore, 10 *Azospirillum* strains did not cause any symptoms in several crop plants when inoculated using conventional infestation methods (Bashan 1996).

Inoculants, new products, and potential industrial uses

The bottom line of every inoculation technology is its successful application under agricultural and industrial conditions. The inoculum formulation and application technology are crucial for the development of commercial *Azospirillum* inoculants. A technique that works mainly under research-laboratory conditions is unlikely to gain success under commercial and competitive markets. Surprisingly, relatively few studies have addressed the application of *Azospirillum* technology, which has been slow to make a significant impact on the inoculation market (Bashan et al. 1995*c*, 1995*d*; Fages 1992, 1994).

Inoculants

After introducing the concept of synthetic inoculants made of alginate into *Azospirillum* technology (Bashan 1986*b*), several commercially oriented studies addressed its application. An optimized process for manufacturing a crop inoculant was developed with an *Azospirillum lipoferum* strain. This process involves the entrapment of living cells in alginate beads and air dehydration. This latter unfortunately eliminated the vast majority of the original cells, but the remaining cells were sufficient to serve as an inoculant (Paul et al. 1993). The highest survival rate was obtained by addition of skim milk and controlled dehydration in air of the alginate beads. Finally, a powdered inoculant was obtained. It was easy to store and handle and can be used in the field as a micro-granule or as a seed coating. The biodegradability insures that there is no environmental pollution (Fages 1990, 1992). Unfortunately, to the best of our knowledge, this French registered inoculant was never released into the market (Fages 1994) .

Prototype equipment for manufacturing alginate beads was designed, one to produce 6-mm granules (Digat 1991) and the second for microbeads (100-200 µm in diameter) using a low air pressure device (Carrillo and Bashan 1996).

Another future possibility for efficient inoculation, valid for plants propagated from tissue culture, is to inoculate the bacteria into the plant cell suspension and regenerate embryos and eventually plants. These plants will probably be inoculated from their onset. As this can be done in a tissue culture laboratory accustomed to sterile and precise work, this will result in even inoculation of the plants (Alcaraz-Melendez 1996). Alternatively, an axenic root tissue culture procedure can be used (de Freitas and Germida 1990).

Vitamins

The production of vitamins by *Azospirillum brasilense* and their liberation were significantly affected by the presence of different carbon sources and the age of the culture. Thiamine, niacin, and pantothenic acid were produced in large quantities (Rodelas et al. 1993), as was riboflavin (Dahm et al. 1993).

Toxic residue degradation

Azospirillum lipoferum was capable of reducing 4-chloronitrobenzene, an aromatic compound used in the manufacturing of pesticides, dyes, explosives, and industrial solvents and an environmental pollutant (Russel and Muszynski 1995).

Lines for future research

During the general discussion of the last international conference on *Azospirillum* and related microorganisms held in Sárvár, Hungary, in 1994, the following issues were recognized by the participants as the major avenues for *Azospirillum* environmental and physiological research in the future: (*i*) coinoculation with other microorganisms; (*ii*) inoculation of woody plants; (*iii*) the role of bacterial and plant surfaces in attachment; (*iv*) *Azospirillum*-plant genotype interaction, specificity, and affinity at the genotype level; and (*v*) environmental fitness (rhizocompetence).

A general analysis of the current knowledge on *Azospirillum* yielded the following conclusions. As the major problems of inconsistency and unpredictability of the inoculation results are not resolved (and not even currently addressed in the literature), the prospects of large scale inoculants (although some small-scale,

new inoculants are already on the market) are as far away as in 1990. A possible agricultural breakthrough might be the use of *Azospirillum* as a helper bacterium together with other beneficial microorganisms. In this role, *Azospirillum* helps the other microorganism perform better and indirectly has a positive effect on plant growth.

Another possible avenue is the development of better bacterial carriers. Twenty years after its rediscovery, *Azospirillum* is still inoculated as it was then: peat inoculants, primarily designed for rhizobia and hardly adjusted to *Azospirillum* needs (Bashan and Carrillo 1996; Fallik and Okon 1996), and inefficient liquid (mostly bacterial culture) inoculants. Even the synthetic inoculants are still in the same initial state for commercialization that were in when they were first introduced (Bashan 1986*b*). Without a practical means of delivery, no beneficial microorganism (including *Azospirillum*) will be commercially successful.

Another exploitation possibility using the knowledge that is accumulating on *Azospirillum* is to produce industrial products based on its physiological features but not related to agriculture. These include production on a large scale of PHB (Okon and Itzigsohn 1992), purification of urban residual water (Gonzalez and Bashan 1996) using its nitrate reductase ability (Ueckert et al. 1991), purification of toxic effluents, production of vitamins, and the breakdown of cellulose in combination with cellulolitic bacteria.

Given our current basic knowledge of *Azospirillum*, the most studied plant-associative bacteria, the prospects of using it for the benefit of mankind are greater than those for other bacteria, but not in the immediate future.

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