

REVIEW ARTICLE

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Interactions of *Azospirillum* spp. in soils: a review

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Abstract This review summarizes and discusses the current knowledge and the, as yet, unanswered questions on the interactions of *Azospirillum* spp. in bulk soil (but not in the rhizosphere). It contains sections on the isolation of these bacteria from tropical to temperate soils, and on their short- and long-term persistence in bulk soil. The interactions of these bacteria with soil particles and minerals such as clay, sand and Ca, and the effect of soil pH, soil redox potential, and the cation exchange capacity of the soil on them is demonstrated. Data is presented on the distribution of *Azospirillum* spp. in soils, on their production of fibrillar material essential for anchoring the cells to soil particles, on the effects of soil irrigation, and of external soil treatments, and on the effect of soil C and C used in bacterial inoculants on the cells. It shows that root exudates possibly govern bacterial motility in the soil. Finally, the effect of pesticide applications, the relationships with other soil microorganisms such as *Bdellovibrio* spp., *Bradyrhizobium* spp., and phages, and the potential use of a community-control model of *Azospirillum* spp. in soil and in the rhizosphere is suggested.

Key words *Azospirillum* · Motility · Plant-growth-promoting bacteria · Root exudates · Soil bacteria

Introduction

Two major approaches dominate contemporary studies on soil microbial ecology: (1) soil microorganisms can be evaluated as an integral part of plant communities, or (2) they can be studied purely for their own sake

with plants acting only as substrates of various kinds (Ohtonen et al. 1997; Wardle and Giller 1997).

So far, *Azospirillum* spp. have been identified mainly as rhizosphere bacteria. The genus contains five species (Table 1). They proliferate in the rhizosphere (soil fraction affected by root activities) of numerous plant species of many families. After establishing in the rhizosphere in sufficient numbers, they usually, but not always, promote the growth of the host plant (Bashan and Holguin 1997a). They are commonly known as plant-growth-promoting bacteria (PGPB; Bashan and Holguin 1998). Although some of their host species are perennials, most are annuals (Bashan and Holguin 1997b). This creates a hurdle for survival from one season to the next, especially when harsh (both high and low) temperatures combined with a long period of drought prevail. Yet, the bacteria survive. Little is known about their saprophytic ecological niche, and whether or when they are able to multiply in soil in the absence of a host plant. In comparison to what is known about their genetics (Holguin et al. 1999; Vande Broek and Vandeleyden 1995), physiology (Hartmann and Zimmer 1994), growth-promotion effects (Bashan and Levanony 1990), agronomic performance and potential (Okon and Labandera Gonzalez 1994), or their interactions with the rhizosphere (Del Gallo and Fendrik 1994), this supposedly important bulk-soil survival phase (i.e. the soil fraction not affected by root activities) is a neglected subject and little is known about it. Despite this, *Azospirillum* spp. do display a range of very efficient physiological mechanisms that may enable them to survive under unfavorable conditions. These include cyst formation (Bashan et al. 1991a; Sadasivan and Neyra 1987), floc formation (Neyra et al. 1995), production of melanin (Givaudan et al. 1993), poly-*b*-hydroxybutyrate synthesis (Okon and Itzigsohn 1992), polysaccharide synthesis (Del Gallo and Haegi 1990), and protection inside ectomycorrhizal fungal spores (Li and Castellano 1987).

Although microorganisms in general, and *Azospirillum* spp. in particular, have shown great promise in in

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Table 1 List of known *Azospirillum* spp.

Species	Reference
<i>A. brasilense</i>	Tarrand et al. (1978)
<i>A. lipoferum</i>	Tarrand et al. (1978)
<i>A. amazonense</i>	Magalhães et al. (1983)
<i>A. halopraeferense</i>	Reinhold et al. (1987)
<i>A. irakense</i>	Khammas and Kaiser (1991)

vitro screening, the expression of their beneficial properties in the natural environment is unpredictable and often disappointing (Bashan and Levanony 1990; Kenney 1997; Stotzky 1997; van Elsas and Heijnen 1990). The fundamental reasons for the disparity between in vitro and in situ results are poorly understood and highly speculative (Hozore and Alexander 1991). In practice, each strain requires detailed field and greenhouse studies of its soil behavior, a laborious, expensive, and mostly uneconomical process, before it can be considered effective and safe for release into the environment. A partial solution, but a more practical one, is the use of laboratory microcosms; results from some of these studies have been shown to be good predictors of survival and activity in the field (Bolton et al. 1991; Prosser 1997; Teuben and Verhoef 1992). However, this approach has been largely neglected in studies on *Azospirillum* spp.

Inoculation of plants with mainly *A. brasilense* and *A. lipoferum* is usually done either by seed inoculation or by application of the bacteria directly to the soil as near as possible to the germinating seedlings (Bashan 1998). This is done because of the relative immobility of many PGPB inoculants in the soil. Albeit not a typical case for *Azospirillum*, the inoculation site is likely to determine its ultimate fate: colonizing the roots and surviving, or dying. During this establishment period, the bacteria are exposed to the natural physical forces and interactions that prevail between soil bacteria and soil particles, like adsorption, encapsulation by clay minerals, and wet and dry regimes of the soil. To overcome these barriers and colonize plant roots, *Azospirillum* spp. must evolve a mechanism to allow movement through the soil. Persistence of *Azospirillum* spp. in the bulk soil is crucial because seed inoculation is impractical in many field applications (e.g. for perennial plants, vegetatively propagated plants, and trees) or when more than one inoculation per season is required (Bashan 1998).

The aims of this review are to highlight what is known of the bulk-soil phase of *Azospirillum* (not including the rhizosphere or the rhizoplane), and to point out feasible lines of research where data are needed.

Isolation from soil

The rhizosphere and root surfaces are *the* sites where one looks for new *Azospirillum* strains (Tyler et al.

1979). *A. lipoferum* (*Spirillum lipoferum*) was originally isolated from a sandy soil (Beijerinck 1925). Subsequently, the various *Azospirillum* spp. were isolated from numerous host roots and rhizospheres (Bashan and Levanony 1990; Bashan and Holguin 1997a, b). Significantly lesser attention has been given to their populations in the bulk soil.

The isolation of *Azospirillum* spp. from bulk soil does not necessarily attest that the cells are physiologically active in situ. On the contrary, nutrient limitation is a major stress factor in soil. Thus, the bacteria endure long periods of no growth and transient periods of growth. They may survive in vegetative or cyst forms until a host plant is available. Isolation of *Azospirillum* spp. from bulk soil has been confirmed worldwide, usually in proportionally lower numbers than from rhizosphere soil. The first screening for this organism showed that about 60% of the soil samples collected in tropical sites (five African countries and tropical Brazil) contained *A. lipoferum* (Döbereiner et al. 1976). This bacterium was isolated from soil of the Amazon region and of Rio de Janeiro State, Brazil (Magalhães et al. 1983), from many other Brazilian soils (Döbereiner 1988), and from rice-field soils in Vietnam (Dung et al. 1995). In temperate zones of Brazil and in the US, the occurrence of *Azospirillum* spp. in soil was significantly lower than in the tropics (Döbereiner et al. 1976). Fields in Belgium had 10-100 times more azospirilla in the rhizosphere than in soil samples. The soil population was low, and never exceeded 10^3 colony-forming units (cfu) g^{-1} soil (De Conink et al. 1988; Horemans et al. 1988). In England, a low incidence of *Azospirillum* spp. was detected in soil compared with higher recoveries from subtropical and tropical soils (Harris et al. 1989). These bacteria persist naturally in the soils of several other countries. They have been found in the Ukraine, in various sandy soils (Maltseva and Volkogon 1984), in Poland in garden soils (Kulinska 1983), in a Japanese soil amended with organic and inorganic manure (Subba-Rao et al. 1984), in root-free soils in Egypt (Nadia et al. 1984; Shawky 1989), in 2 alluvial, virgin soils from Somali (Favilli et al. 1988), and from highly saline soils in India (Jena et al. 1988; Tilak and Murti 1981). However, when eight plant-free soils from Brazil and Canada were tested for indigenous azospirilla, none was found (Germida 1986).

One uncertainty was involved in many, but not all, of these studies. The isolation procedures did not rule out the possibility that these bacteria were actually present in plant debris in the soil and not in the inorganic soil fraction, as many of these soils were under cultivation or previously covered with vegetation. Rarely were dry, sieved, and soils stored for a long period used for isolation of *Azospirillum* spp. Thus, there is no conclusive evidence that *Azospirillum* spp. can survive in, and be isolated from, organic debris-free soils.

Short- and long-term persistence in soil

Several independent factors complicate the collection of meaningful data on survival of PGPBs in soil:

1. The variation between soil is great, and every site may be different, even on a microbial scale (van Elsas 1992). Furthermore, in many countries, soil types have not been assessed adequately, if at all.
2. Few studies on the types and distribution of micro-environments in a particular soil have been done, although these are widely recognized as being crucial for bacterial survival (Stotzky 1997).
3. Thorough studies of soil factors affecting the survival of PGPB are scarce (Hartel et al 1994; Stotzky 1997).
4. The research and development sector of the inoculation industry has largely ignored the importance of basic soil ecological studies that may show what controls the fate of inocula. Commercial *Azospirillum* spp. Inoculation technology is a good example of this (Fage 1992).

Contradictory data on the survival of *Azospirillum* spp. in soil have been published since the start of *Azospirillum* spp. agricultural research. Several examples illustrate this. On the bright side, *Azospirillum* spp. were detected in Nigerian soil samples stored in a United Kingdom laboratory for at least 10 years (Harris et al. 1989). In a Brazilian garden soil, when *A. lipoferum* was introduced, its population declined rapidly to less than 10^2 cfu g⁻¹ soil, but later recovered and remained constant at about 10^3 cfu g⁻¹ soil for 120 days (Oliveira and Drozdowicz 1988). Inoculated *A. brasilense* Cd and Sp-245 survived well in Brazilian soils in addition to their survival on roots (Baldani et al. 1986). In Danish agricultural soils under continuous barley cultivation, *Azospirillum* spp. were present at about 10^4 cfu g⁻¹ (dry soil) in sandy-loam soil and at 10^5 cfu g⁻¹ (dry soil) in a coarse sandy soil (Idris et al. 1981). Inoculation of a temperate isolate into wheat cultivated in England resulted, apart from survival in the roots, also in survival of 14 months in the soil, albeit in low numbers of approximately 10^2 cfu g⁻¹, which was enough to allow the bacteria to overwinter (Harris et al. 1989).

More meaningful results were obtained when *A. brasilense* and *A. lipoferum* were inoculated into the soil in laboratory experiments to measure their population dynamics. In a comparison between the levels of *A. brasilense* in the rhizosphere and bulk soil of a heavy-textured tropical soil from Martinique (French West Indies), *A. brasilense* numbers were 300 times higher in the rhizosphere than in the bulk soil. Despite this, soil-fraction analysis revealed that *A. brasilense* had a preference mainly for the macroaggregates of the soil, where it sustained a high population level of over 10^6 cells g⁻¹ soil fraction, and to a lesser extent for the fine clay particles. Yet, these numbers represented only 0.18% of the total bacterial counts of these fractions (Kabir et al. 1994). The population dynamics of *A. lipo-*

ferum inoculated into γ -sterilized soil were similar in maize-planted and nonplanted soil. The population increased with time in both soils during 20 days of incubation (Steinberg et al. 1989). Similarly, when a Tn5 mutant of *A. brasilense* was introduced into an unplanted, sterilized soil, the number of cells remained at a constantly high level over a period of 100 days (10^7 cfu g⁻¹), but dropped sharply in unsterilized soil, during the same period, to 10^3 cfu g⁻¹ (Christiansen-Weniger 1992; Christiansen-Weniger and Van Veen 1991).

On the negative side, in the USA, Albrecht et al. (1983) observed that even after substantial inoculation of a forage crop during sowing, the *A. brasilense* Cd population declined rapidly in only 15 days, and had nearly vanished in the soil after 25 days. Continuous monitoring showed the population fell below 10^2 cfu g⁻¹ soil up to 6 weeks after inoculation and later disappeared. *A. brasilense* Cd cells could not be detected in the next crop season. Using the same strain, Smith et al. (1984), reported a sharp decline in the soil population after 3 weeks, reaching a population of 10^2 cfu g⁻¹ soil, whereas 5 weeks later the bacteria had almost disappeared. This behaviour of *Azospirillum* spp. in the soil is similar to that of fecal coliform bacteria in soil (Elliott and Ellis 1977).

More detailed laboratory experiments showed that in Israeli soils, *Azospirillum* spp. survived less than 15 to 20 days in light-textured sandy soils and 9 days in heavy-textured soils in the absence of plants (Bashan and Levanony 1987, 1988a). In wet quartz sand, *Azospirillum* inoculated at high numbers (10^8 cfu g⁻¹ sand) could not be detected for more than 20 days (Bashan et al. 1991b). Early exponential, or exponential growth phases of *Azospirillum* spp. when inoculated into soil resulted in a higher, stabilized, population level and a lower death rate than when early stationary-stage or stationary-phase inocula were used. When inoculated into sterilized soil extracts, early exponential-phase bacteria showed a shorter time lag and a higher multiplication rate than bacteria from the later growth phases (Vandenhove et al. 1993).

Although this study showed that the physiological stage of *Azospirillum* may have an effect on their general persistence in soil, a change in the physicochemical properties of the soil may have a much greater effect on the survival of the bacteria in soil than changes in the bacteria themselves (Stotzky 1997). Some examples corroborate this assumption. A survival study of inoculated (10^7 - 10^8 cfu g⁻¹ soil) *A. brasilense* Cd in Brazilian and Canadian soils showed that in most soils the population declined rapidly within the first 2 weeks and settled at, a level of about 10^5 cfu g⁻¹ soil. Nutrient amendment increased the bacterial population for several days, and then it returned to its usual soil population level (Germida 1986). Similarly, as reported earlier by Smith et al. (1984) for *Azospirillum* spp. and for *Flavobacterium* sp., survival rates increased with the soil water-holding capacity and by amendment of the soil

with various sugars (Mawdsley and Burns 1994). The survival of *A. brasilense* Cd and Sp-245 in 23 types of plant-free sterilized soils obtained from a wide range of environments in Israel and Mexico was evaluated. As is common, large numbers of *A. brasilense* were detected in all the rhizospheres tested, regardless of soil type, bacterial strain, and the origin of the soil. However, survival of *A. brasilense* in root-free soil (bulk soil) differed from that in the rhizosphere and was mainly related to the geographical origin of the soil and not to the original level of aridity of the soil. In Israeli soils from arid, semiarid, or mountainous regions, viability of *A. brasilense* rapidly declined or the population disappeared completely below detectable levels within 35 days after inoculation. In contrast, populations in the arid soils of Baja California (Mexico) remained stable or even increased during the first 45 days after inoculation. In soils from nonarid central Mexico, viability slowly decreased with time (Bashan et al. 1995a).

Though *Azospirillum* spp. might survive in some soils and not in others, there are several inherent difficulties related to the methodology used. Several researchers who attempted to reisolate inoculated *Azospirillum* spp. used only enriched-culture methods. This procedure reveals little or nothing about the abundance of the organism or its metabolic activity either in the rhizosphere or in bulk soil. Other researchers did not take precautions in order to sample bulk soil alone, and actually sampled the rhizosphere where *Azospirillum* is abundant. Another difficulty emerged from studies with sterilized soil preparations used for direct inoculation. Perhaps sterilization (both autoclaving and γ -irradiation) makes assimilable organic compounds, dead cell debris, and released organic compounds more available, thus increasing detectable bacterial numbers.

It can be concluded that *A. brasilense* is a rhizosphere bacterium that survives poorly in some bulk soils (but does well in the rhizosphere). However, like other soil bacteria, on being introduced into soil, it may turn into unculturable forms (Refer Bakken 1997). This, however, has not been shown for *Azospirillum* spp.

Interactions with soil particles

In soil

Most indigenous soil bacteria are adsorbed permanently on soil particles (Hattori and Hattori 1976), and the majority of the cells are unculturable (Refer Bakken 1997). As explained above, in many PGPBs (and sometimes in *Azospirillum* spp.), there is a general decline in bacterial populations following soil inoculation. These bacteria are probably localized in more open spaces within the soil than indigenous populations. There, they are affected to a greater extent by nutritional and physicochemical soil factors. Introduced bacteria, like

Azospirillum spp., are also affected by the same stress factors as indigenous bacteria, but to a greater extent than the latter which are adapted to these conditions (van Elsas and van Overbeek 1993).

The ability of different soils to adsorb introduced bacteria depends primarily on the physicochemical composition of the soil particles and to a lesser extent, on the bacterial species present or the bacterial growth conditions prior to inoculation. The degree of adsorption is broadly related to the soil surface area and the particle surface charges. Hence, clay and organic matter particles are the most important soil components. Bacteria, clays, and organic matter particles possess a similar net negative surface charge that theoretically prevents contact between them. This raises the question: how do soil particles adsorb bacteria? The answer is that clay particles may possess positively charged edges to which bacteria can adsorb.

Several detailed laboratory studies of adsorption to bulk soil of *A. brasilense* suggests that the same criteria apply to this bacterium as to other soil bacteria. *A. brasilense* Cd adsorbed strongly to light-textured and heavy-textured soils but only slightly to quartz sand. In the latter it was easily washed down to a depth of 100 mm below the inoculation site (Bashan and Levanony 1988a), as were *Lactobacillus* spp. (Huysman and Verstraete 1993). An increase in the clay or organic matter content of the soil to >5% increased adsorption to a level similar to that of many other species of soil bacteria. Application of bacterial attractants to only part of the soil caused the bacteria to migrate and to accumulate at the site of the attractant. This indicates that motility induced by attractants is stronger than adsorption to the soil particles. Drying the soil or an increase in soil pH decreased adsorption. A decrease in soil pH, which increases the positive-charge density on the edge of clay particles, or flooding the soil, enhanced adsorption (Bashan and Levanony 1989). The pH effect on soil adsorption is strongly influenced by plants because of the modification of the soil solution pH by plant exudates. Growing wheat roots (a common *Azospirillum* spp. host) decrease soil pH by proton extrusion (Bashan et al. 1989). *A. brasilense* cells colonizing wheat or soybean roots further enhanced proton extrusion from the roots (Bashan 1990; Bashan and Levanony 1991), lowering the soil pH even further. Consequently, it can be hypothesized that the partial failure of introduced *Azospirillum* spp. to colonize some roots may be attributed to an increased adsorption capacity of the soil which reduces the number of bacteria available for root colonization.

An additional soil variable that may affect adsorption of *Azospirillum* spp. is the cation exchange capacity (CEC) of the soil. The higher the CEC, the higher the percentage of adsorbed cells (Govindarajan and Purushothaman 1989). Finally, the soil redox potential which directly affects N_2 fixation by *Azospirillum* spp. may also affect adsorption (Charyulu and Rajaraman Rao 1980).

The distribution of soil bacteria within soil layers is usually not homogeneous. Their relative penetration depends on whether they are strongly adsorbed to the soil particles, or whether they are restricted in their movement through habitable pore spaces and capillaries, or the availability of water films. The distribution of *A. brasilense* Cd was different from that of other soil bacteria, as it was homogeneous and restricted to the site of application. The cells were adsorbed on the upper fraction (20-30 mm) of the soil profile. Live bacteria were adsorbed in higher numbers than dead bacteria. The physical adsorption forces combined with bacterial protein bridges formed between the bacterium and the soil particles strongly anchored *A. brasilense* Cd. These holdfasts counteracted the forces created by flowing water. External washing, even in excess (equivalent to 3000 mm of rainfall), recovered relatively few cells (Bashan and Levanony 1988a). Field evidence that supported the findings of *A. brasilense* attachment to large soil particles was its persistence in macroaggregates of tropical bulk soil (Kabir et al. 1994).

In sand

It is an advantage for any PGPB to remain in the vicinity of the root and not be washed away. Additionally, sand is a far less complex growth substrate than that of most soils. However, although providing atypical conditions with respect to most soils, sand cultures allow a specific soil variable to be studied without interference by others.

If it is to survive and affect plant growth, *A. brasilense* Cd inoculated into sand should be applied to the particles in such a way as to prevent its removal from the root zone by irrigation water. Incubation of *A. brasilense* Cd in quartz sand (small surface area, negligible clay and organic matter content, yielding minimal surface-charge properties) resulted in its attachment to sand particles by protein bridging, a network made up of various sizes and shapes of fibrillar material which was single-stranded or multistranded. These fibrils prevented the cells from being washed away and were on all sides of the bacterial cell. However, only 56% of the applied bacteria adsorbed (Bashan and Levanony 1988a). Similarly, Fehrmann and Weaver (1978) demonstrated the attachment of rhizobial species to silt particles, but by a network of fibrillar polysaccharides. Attachment of *A. brasilense* Cd to sand was relatively weak, and depended on the presence of living bacterial cells and on laboratory bacterial culture conditions prior to inoculation. Dead *Azospirillum* spp. did not attach to sand, contrary to the effect observed in fine-textured soil. When nutrients were limiting, the cells could not support the production of excess fibrillar material. But, as a result of the addition of nutrients at low concentrations, such as fructose or malate with NH_4Cl , root exudates enhanced attachment to sand, albeit weakly. The bacteria could be desorbed by washing,

releasing in the process proteinaceous compounds into the sand. Substances to which the bacteria are resistant, such as streptomycin, did not affect bacterial adsorption and multiplication. Nonetheless, addition of protease, EDTA, various bacterial inhibitors, or exposure to a high temperature (42 °C) of sand-attached *A. brasilense* Cd significantly reduced attachment. Hence, the nature of the fibrillar material is probably proteinaceous (Bashan and Levanony 1988b). However, one should be aware that this is only circumstantial evidence showing that substances inhibiting protein synthesis or proteases decreased attachment and caused a temporary increase in the protein content of the sand. Further evidence of the importance of fibrils in attachment was found when a wild-type *A. brasilense* Cd was inoculated into sand in comparison with an attachment deficient mutant. The mutant produced no detectable fibrils. Although similar populations of both strains developed in the sand, the attachment ratio between wild-type *A. brasilense* Cd and its attachment-deficient mutant was 4:1 (Bashan et al. 1991b). It appears that fibrils are not essential for bacterial multiplication in sand, but are for attachment.

Another adverse factor affecting attachment to sand was agitation. Immediately after application to sand, agitation reduced the attachment of *Azospirillum* spp. but increased their multiplication significantly. Attachment under microaerophilic conditions was lower than under aerobic conditions and depended on the amount, quality, and composition of the available nutrients. The richer the mixture, the greater the attachment (Bashan et al. 1991b; Bashan and Levanony 1988b; Levanony and Bashan 1991). Thus, by slightly manipulating the nutritional status of the sand, one can markedly change the extent of attachment.

The effects of single soil variables

The effects of single soil variables, such as soil texture, on survival and activity of soil bacteria are well known (Foster 1988; van Elsas et al. 1991; van Elsas 1992; van Elsas and van Overbeek 1993). In Israeli and Mexican soils, clay content, N, organic matter, and water-holding capacity were positively correlated with *A. brasilense* viability (Bashan et al. 1995a). In an unplanted, inoculated temperate climate soil or washed sea-sand, nitrogenase activity was low, but increased by 60% when malic acid was added as a C source. There was a peak of nitrogenase activity 3 days after inoculation, perhaps because of the release of readily available organic C resulting from the γ -irradiation of the soil (Christiansen-Weniger and van Veen 1991). Viability of the five known species of *Azospirillum* spp. in two "artificial" soils containing the same major components as natural soils from Israel was almost identical to that of the natural soils (Bashan and Vazquez 1999, unpublished). No single abiotic soil variable mentioned above was responsible for survival. However, when several of

these minor factors were acting in concert, they increased survival. Variables that by themselves had a negative effect on *Azospirillum* spp. viability in soil included a high percentage of CaCO₃ and sand content. The percentage of silt, P and K, electrical conductivity, pH, and C/N ratio had no apparent effect on bacterial viability. However, nutrient sources or addition of organic matter did. Removal of plants growing in soils greatly reduced the survival of *A. brasilense* remaining in these soils. In as little as 15 days, the bacterial population in the plant-free soil began to decline rapidly, reaching undetectable levels about 60 days after inoculation, depending on the *Azospirillum* species (Bashan et al. 1995a,b; Bashan and Vazquez 1999, unpublished). It is not yet known whether the negative effect of intermediate-sized (neck diameter, 6-30 µm) pores on bacterial survival, resulting from protozoan grazing (Wright et al. 1995), and improved bacterial survival in small-necked pores (Hassik et al. 1993), also applies to *Azospirillum* spp. populations in the soil.

Several conclusions can be drawn from the above studies. In soil containing clay and organic matter, these rhizosphere bacteria share some soil adsorption features with many other soil bacteria (Marshall 1980), e.g. live and dead bacterial cells are irreversibly adsorbed to soil particles and rinsing or other external physical forces have only marginal effects on bacterial adsorption. To survive in marginal substrates such as sand, the bacteria should be metabolically active, permitting production of external fibrillar materials that is an essential condition for their survival in sand. One should be aware that this type of active attachment of *A. brasilense* to sand contrasts with that of most soil bacteria that survive in soil in a permanently adsorbed state. Soil abiotic variables, only when acting together, have an effect on the survival of *Azospirillum* spp.

Interaction with organic matter and plant debris

The laboratory experiments described above showed that amendment of soil with organic matter enhances adsorption and survival of *Azospirillum* spp. However, there is also evidence from the field on the effects of organic matter on *Azospirillum* spp. in soil that contradicts findings from the laboratory.

Azospirillum spp. (and most PGPB) inocula frequently contain organic matter (Bashan 1998). In India, amending garden soil, which supported only a limited *A. brasilense* population, with different types of organic matter such as straw composts (to create an inoculum carrier) increased the amount of *A. brasilense* in the mixtures (Negi et al. 1987; Sadasivam et al. 1986). Soil or coal amended with organic nutrients supported an initial increase in the *Azospirillum* spp. population, but it declined later to a level comparable to that of the unamended carrier (Joseph and Dube 1988). In the USA, survival of *A. brasilense* in inocula carriers of peat and sand was monitored. After the common initial

decline, most populations remained stable for about 60 days. Carriers with the highest peat content (1-3%) had the largest populations of *Azospirillum* spp. (Albrecht et al. 1983). In India, addition of rice straw to flooded soils enhanced the population of *Azospirillum* spp. (Charyulu and Rajaramamohan Rao 1980). In Israel, however, the number of *A. brasilense* Cd in sand mixtures amended with organic matter decreased as the organic matter in the sand increased, and the effect of *A. brasilense* on plants was also seen to diminish when the proportion of organic matter exceeded 1% (Fallik et al. 1988).

While it was earlier explained why organic matter favoured the survival and persistence of *Azospirillum* in soil, theories for the negative effects of organic matter might be that at high organic matter concentrations, total bacterial numbers reached 10⁷-10⁸ cfu g⁻¹ sand and other bacteria competed with the *Azospirillum* inoculated into the soil. Alternatively, the organic matter may have provided enough nutrients for the plant and the effect of bacterial inocula was therefore obscured.

Effect of pesticides in soils

Azospirillum spp. are frequently inoculated into heavily pesticide-treated fields. Nevertheless, their interactions with pesticides have hardly been addressed and mostly studied in vitro (for review see: Bashan and Holguin 1997a). Even less information is available about the possible effect of pesticide accumulation in the soil on *Azospirillum*. In rice cultivation, there was indirect involvement of soil microorganisms, including *A. lipoferum*, in the degradation of the insecticide carbofuran in flooded soils (Venkateswarlu and Sethunathan 1984). The insecticide even increased N₂ fixation of several isolates (Jena et al. 1992). However, this meagre information is insufficient for meaningful conclusions to be drawn.

Motility in soil

For application purposes, because inoculation of the entire root system of plants is impractical, *Azospirillum* spp. cells are incorporated into various types of inocula carriers, which, in light of their agrotechnical nature, cannot ensure that the bacteria will always encounter the emerging roots (Bashan 1998). As a consequence, *Azospirillum* spp. movement from the inoculation site to the root is essential if root colonization is to occur. This movement, from a few microns to several centimetres, depends on root exudates and occurs in an environment of fierce competition with other soil microflora that are also seeking nutrients and root colonization sites on the growing roots. Hence, the motility of *Azospirillum* spp. in the soil can be considered an important trait.

Motility is a major taxonomic property of the genus *Azospirillum* (Tarrand et al. 1978). Of the many studies

of chemotaxis of *Azospirillum* spp., almost all were done in vitro (for a review see Zhulin and Armitage 1992) and a very few in soil using a single strain, *A. brasilense* Cd. The main factor affecting the movement of *A. brasilense* Cd towards wheat seedlings grown in soil was soil moisture, with maximum movement near and above field capacity. This is because soil is comprised of a series of discontinuous surfaces and water films that restrict and prevent direct migration except under extremely moist conditions. Of secondary importance was the soil type; the coarser the soil texture, the higher was the rate of migration. Plant development also affected motility positively. Migration was characterized as a band of bacteria migrating through the soil towards the plant roots. Nearly all the bacteria migrated simultaneously. No cells could be detected in a given patch of soil 48 h after the bacterial population had migrated from that site. This nonspecific migration was significantly stimulated by glycine and aspartic acid, which are known root exudates (Bashan 1986a).

In light-textured-soil trays, soil columns, and in the field, *A. brasilense* Cd motility was essential for the colonization of entire root systems, and of neighbouring plants (in the field). However, this occurred mainly in the rhizosphere because roots of adjacent plants formed a continuous system. Migrating distances in the rhizosphere are measured in millimetres, although *A. brasilense* Cd can migrate in root-free soil for 30 cm solely along a root-exudate gradient (Bashan and Levanony 1987). These results with *A. brasilense* Cd supported the findings that nonmotile mutants of biocontrol/PGPB pseudomonads were impaired in their ability to move towards seeds compared to the motile wild type (Scher et al. 1985).

In view of the above, passive dispersion by percolating water, especially in semiarid conditions where *Azospirillum* spp. showed their best performances (for a review see: Bashan and Levanony 1990), cannot adequately explain how *Azospirillum* spp. colonize the entire root system of a plant. This is especially true when *Azospirillum* spp. cells on the root surface are permanently anchored by fibrillar material (Levanony et al. 1989; Michiels et al. 1991). Therefore, it is plausible that bacterial motility in soil is responsible for this dispersion. The root-to-root migration (wheat and soybean) of *A. brasilense* Cd in quartz sand and light-textured soil was compared by using a motile wild-type (Mot^+) and a motility-deficient strain (Mot^-). Mot^+ cells moved through sand and light-textured soil from inoculated roots to noninoculated roots. The effects of attractants (derived from common root exudates) and repellents were the primary factors governing motility (Bashan and Holguin 1994). Movement between roots and dispersion of *A. brasilense* Cd in the field and in soil trays in a growth chamber showed that in the field, Mot^+ cells moved from inoculated roots to noninoculated roots of either wheat plants or weeds growing in the same field plot, but the Mot^- did not. In plant-free, water-saturated soils, either in soil columns or in the

field, both strains remained at the inoculation site and did not move, though they moved between weeds and wheat freely (Bashan and Holguin 1995).

In conclusion, the increased survival of *Azospirillum* spp. in unplanted soil with the addition of root exudates indicates that in natural environments the availability of a C source is an important limiting factor in bacterial activity. This dependency on root exudates is, for example, the reason for the variation in rhizosphere N_2 fixation that is found in different varieties of the same plant species (Krotzky et al. 1988; Newman 1985). Root exudates allow the growth of an active bacterial population, which starves when these nutrients are depleted. Motility of wild-type strains in the soil towards root exudates appears to increase the survival probabilities of an organism like *Azospirillum*, which survives poorly in some soils and is dependent on plants for survival. The soil surface is inhospitable for bacteria as it dries. However, it is advantageous if the bacterial cells can move to different plant species or neighbouring plants when their original host dies. Bacterial motility in the soil might play a general role in enabling *Azospirillum* spp. to access sites where more stable colonization might later occur.

Interaction with other soil microorganisms

Unlike within the rhizosphere where *Azospirillum* spp. may comprise up to 10% of the bacterial population (for a review see: Del Gallo and Fendrik 1994), their proportion in bulk soil, even in rich tropical soils, is less than 0.2% of the total bacterial population (Kabir et al. 1994). As a minority, it is plausible that they should be influenced by the soil microbial community and microfauna. Only several examples of these interactions can be found in the literature.

The general interactions of *A. brasilense* Cd with unidentified microflora in physicochemically defined microcosms showed that *A. brasilense* Cd comprised a larger proportion of the community in a mixed culture with compost microflora than when mixed with soil microflora. Furthermore, community-level interactions, rather than their capability to fix N_2 , controlled their proliferation (Janzen and McGill 1995). Inoculation of anaerobic soil with *Azospirillum* spp. affects the redox potential and consequently microbial activity dependent on high redox potential, like denitrification (Pidello et al. 1993). *A. lipoferum* produced bacteriocin(s) in culture but not in soil, and retained its capacity to produce the antibiotic when returned to the culture (Oliveira and Drozdowicz 1988).

Soil commonly reacts as a "biological buffer", hence, any change in its microbial population is only temporary. The application of PGPB, particularly under wet conditions, increases the population of nearby microorganisms which "prey" on the applied PGPB until they are extinct. There was a temporary reduction in the number of microbial competitors after applying inhibiting substances, to which *A. brasilense* Cd is resistant, to

inoculated soil cultivated with wheat. Four successive inhibitor applications, at weekly intervals, significantly reduced the total soil and rhizosphere bacterial populations. This inhibitory effect lasted as long as 2 weeks, after which the bacterial population returned to its initial level. By that time, however, the *A. brasilense* Cd population in the soil and wheat rhizosphere had increased significantly. This resulted in better root colonization and a significant effect on plant growth and yield (Bashan 1986b). This approach demonstrated the possibility of temporarily depressing natural competitors in soil and the potential use of a community-control model in soil and the rhizosphere. However, the inhibitory substances used in the study are not practical because they are expensive and are not licensed for use in agriculture.

Little is known about the interactions of *Azospirillum* spp. with specific soil microorganisms besides its being parasitized by *Bdellovibrio* spp. and bacteriophages and it being synergistic with *Bradyrhizobium*. *Bdellovibrio* that prey on *A. brasilense* were isolated from two Brazilian soils that also were stored air-dried for 2 years. Direct assay of these soils did not yield any *Bdellovibrio*. However, inoculation of soil with *A. brasilense* Cd or Sp-7 and nutrients stimulated growth of indigenous *Bdellovibrio*. *Bdellovibrio* preferred *A. brasilense* Cd cells as prey rather than strain Sp-7, which are almost physiologically identical (Germida 1987).

One Brazilian soil contained an *A. brasilense* Cd bacteriophage. The phage survived permanently in the soil at undetectably low levels. It required a host cell population of at least 10^3 g^{-1} to multiply. The phage population in the soil closely followed *A. brasilense* Cd numbers. Consequently, both could be manipulated by the amendment of soil with nutrients which supported bacterial host-cell growth and stimulated a phage burst for several days only. Later, the phage returned to its undetectable survival level in the soil. When nutrient amendments failed to stimulate the growth of *A. brasilense* Cd, only external inoculation with fresh bacterial host cells together with nutrients resulted in a new burst in the phage population (Germida 1984, 1986). These results indicated that both parasites persist in soil unassociated with *A. brasilense* Cd host cells. Their numbers increase only when the host is present either as a result of being inoculated into the soil or when the bacterial population is increased by the addition of nutrients.

Studies on the saprophytic competence of strains inoculated into soils have been used to examine the regulatory factors that control the population dynamics of these strains, like the denitrification potential. Co-inoculation of *A. lipoferum* and *Bradyrhizobium japonicum* (both can denitrify under saprophytic conditions) into soil produced a higher denitrifying enzyme potential than inoculation with a single strain. The growth of *A. lipoferum* was unaffected by the presence of *B. japonicum*. However, the presence of *A. lipoferum* strongly stimulated the growth of *B. japonicum*. The

very high denitrifying potential obtained suggests that although *B. japonicum* did not affect the *A. lipoferum* growth rate, it was probably able to stimulate its denitrifying potential (Steinberg et al. 1989).

Conclusions and questions

Although the various *Azospirillum* species known are typical rhizosphere bacteria and no important phase in the bulk soil has yet been found for this genus, it shares some of its adsorption characteristics with many soil bacteria. However, unlike many other soil bacteria, *Azospirillum* spp. are not limited by adsorption to soil particles as they are able to move through the soil to target plants, similarly to some biocontrol PGPB pseudomonads (Scher et al. 1985).

Some exploratory lines of research should cast more light upon obscure details of the interactions of *Azospirillum* spp. in soil:

1. Do *Azospirillum* spp. have an unculturable phase in soil? Is the failure to isolate *Azospirillum* spp. from many soils due to this?
2. Are other species and strains of *Azospirillum* besides *A. brasilense* Cd motile in soil?
3. Do *Azospirillum* spp. interact with specific soil microflora in their soil saprophytic phase? Upon introduction, do they fall prey to other bacteria in the soil? Does it contain soil competitiveness features that are expressed also in situ?
4. What are the effects of pesticides on the soil phases of *Azospirillum* spp.?
5. Is it a common mistake to inoculate the bacteria directly into the soil? Perhaps application methods employing direct root colonization would be more fruitful?
6. What are the relationships, if any, between the erratic effects of *Azospirillum* spp. on plant yield and their survival in soil?
7. Do *Azospirillum* spp. overwinter? And, if so, where?
8. Except in the tropics, do *Azospirillum* spp. survive as major components of the soil microflora?
9. Does organic matter affect the survival of *Azospirillum* spp. in soil? Are root exudates the main driving force behind *Azospirillum* spp. activities in soil?
10. Why do *Azospirillum* populations decline quickly in some soils and persist in others? What are the effects of particular soil components on their persistence?
11. Finally, should *Azospirillum* spp. be considered rhizosphere bacteria or bulk-soil bacteria, or both?

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