REVIEW/SYNTHÈSE

Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture[1]

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The genus Azospirillum

The first species of *Azospirillum* was isolated by Beijerinck (1925) from N-poor sandy soil in the Netherlands and was originally named *Spirillum lipoferum*. This bacterium was later isolated from soil (Schroder 1932) and from dried seaweed in Indonesia (H. C. Derx, unpublished, 1949, cited in Becking, 1963) and as a phyllosphere bacterium of tropical plants (Becking 1982). J. Döbereiner and J. M. Day (1976. First International Symposium on Nitrogen Fixation, Pullman, WA. Edited by W. E. Newton and C. J. Nyman. Washington State University Press, Pullman, WA. pp. 518-538) isolated the bacterium and were the first to report that it was widely distributed in the rhizosphere of several tropical grasses. Since then, *Azospirillum* has been isolated from the roots of numerous wild and cultivated grasses, cereals, and legumes, and from tropical, subtropical, and temperate soils worldwide (Bally *et al.* 1983; Döbereiner *et al.* 1976; Hill *et al.* 1983; Horemans *et al.* 1988; Kosslak and Bohlool 1983; Ladha *et al.* 1987; Lamm and Neyra 1981; Li and Castellano 1987; Nur *et al.* 1980b; Rao and Venkateswarlu 1982, 1988; Sundaram *et al.* 1988; Tyler *et al.* 1979; Wong *et al.* 1980).

Tarrand *et al.* (1978) proposed *Azospirillum* as the genus and distinguished two species: *Azospirillum brasilense* and *A. Lipo-*

ferum, based on physiological and morphological differences between various strains and on DNA homology experiments (Falk *et al.* 1986). Later, two additional *Azospirillum* species were described: *A. amazonense* (Falk *et al.* 1985; Magalhães *et al.* 1983), isolated from many grasses in the Amazonian area of Brazil, and the salt-tolerant species *A. halopraeferans,* associated exclusively with roots of Kallar grass (Reinhold *et al.* 1987). However, most strains are referred to as either *A. brasilense* or *A.* lipoferum (Krieg and Döbereiner 1986).

Effect of Azospirillum inoculation on plants

Inoculation of plants with *Azospirillum* can result in a significant change in various plant growth parameters, which may or may not affect crop yield. Descriptions of plant responses to inoculation comprise most of the *Azospirillum* literature. The exact mechanism(s) of action of *Azospirillum* on plants has not yet been fully elucidated.

Most studies of the *Azospirillum*-plant association have been conducted on cereals and grasses (Patriquin *et al.* 1983) and only a few other plant families have been investigated (Bashan *et al.* 1989b, 1989c; Crossman and Hill 1987; Kolb and Martin 1985; Saha *et al.* 1985). The following aboveground plant responses to *Azospirillum* inoculation in cereals and noncereal species were often reported: increases in total plant dry weight, in the amount of N in shoots and grains, and in the total number of tillers, fertile tillers, and ears; earlier heading and flowering time; increased number of spikes and grains per spike; increased grain weight; greater plant height and leaf size; and higher germination rates (Albrecht *et al.* 1981; Baldani and Döbereiner

1980; Bashan 1986*a*; Bouton and Zuberer 1979; Bouton *et al.* 1979; Cohen *et al.* 1980; Hegazi *et al.* 1983; Kapulnik *et al.* 1981 a; Mertens and Hess 1984; Millet and Feldman 1986; O'Hara *et al.* 1981; Pacovsky *et al.* 1985 b; Schank *et al.* 1981, 1985; Warembourg *et al.* 1987; Yahalom *et al.* 1984). In addition, a marked inoculation effect on development of the root system such as on root length and volume have frequently been observed (discussed later). Visible changes in plant growth attributed to *Azospirillum* inoculation were rarely observed in cereals, especially under field conditions, and only detailed statistical analyses revealed significant increases. However, in a few cases, such an enhancement in vegetative growth of cereals was observed in the field (Kapulnik *et al.* 1983). Visible enhancement in the growth of several vegetable plants has been also recently reported (Bashan *et al.* 1989b).

The effect of *Azospirillum* inoculation on the total yield increase of field-grown plants generally ranged from 10 to 30% (Kapulnik *et al.* 1981 *c*, 1987; Rao *et al.* 1983; Watanabe and Lin 1984). A few reports indicated extremely higher values, 50-270% over noninoculated controls. Even moderate yield increases (up to 20%) attributed to inoculation with *Azospirillum* are considered commercially valuable to modern agriculture, if obtained consistently. However, the incidence of positive results may not be frequent enough to enable commercialization of the bacterial preparation; negative or no-effect results of inoculation were rarely reported (Albrecht *et al.* 1981; Harris *et al.* 1989; Smith *et al.* 1984*a*, 1984 *b*). The reports of very high yield responses were frequently due to low-yielding control plots, resulting from undetermined field conditions (Jagnow 1987). Proper evaluation of "plant yield response" reports is difficult because of lack of statistical analysis of data combined over different growing seasons and locations, i.e., results of different experiments are analysed separately. In addition, many of these experiments could not be repeated properly as a result of the lack of essential technical details describing the methods. Okon (1985) evaluated the worldwide success of *Azospirillum* inoculation and concluded that positive effects on yield were obtained in approximately 65% of all field experiments. Yield increases due to inoculation were reported in 75 % of all experiments using summer cereals and only in 50% of the experiments using spring wheat (Smith *et al.* 1984*b*; Schank and Smith 1984). Recently, about 70-75% of all pot experiments in cotton and several vegetables resulted in yield increase (Bashan *et al.* 1989*b*).

Two basic variables that contribute to the complexity of plant yield response to inoculation are the plant cultivars, which often show differential response to inoculation (Bouton *et al.* 1979; Millet *et al.* 1986), and the level of N fertilization. The highest yield increases were obtained when the levels of N fertilization were suboptimal for maximum yield (Kapulnik *et al.* 1981 *b*; Lau-Wong 1987; Mertens and Hess 1984; O'Hara *et al.* 1987). Therefore, *Azospirillum* inoculation was considered a partial substitute for N fertilization. However, contradictory data (Bashan *et al.* 1989b; Millet and Feldman 1986) showed that yield was increased by inoculation even under high levels of N fertilization and that N can influence the number of bacteria in the rhizosphere (Kolb and Martin 1988).

The crucial problem of most greenhouse and field experiments conducted thus far is the inconsistent plant response to inoculation regardless of the plant species. Results have been erratic and success of a field experiment is never ensured (Patriquin *et al.* 1983). Numerous reports from popular agricultural literature and commercial companies indicate that identi-

cally designed field experiments, conducted simultaneously under similar environmental conditions, have failed to produce the expected similar yield results. Experiments failed to improve repeatability of yield response through agrotechnical-management, host-plant, bacterial-strain, or improved-inoculation techniques (Schank and Smith 1984). The development of a reliable and consistent inoculation technology determines whether the future of *Azospirillum* plant interaction will be used only as a biological model for fundamental studies of plant beneficial bacteria associations or will have a significant impact in future agricultural production. The problem may be overcome by a better understanding of the following factors: mode of action of *Azospirillum* in promoting plant growth; root colonization by *Azospirillum*; interaction and competition of *Azospirillum* with other microorganisms in the rhizosphere and the role of the host plant in these interactions; genetics of the bacteria-plant association and subsequent genetic manipulation of the bacteria; activity and survival of *Azospirillum* in the rhizosphere as affected by various environmental factors and including the inconsistency in plant response as a variable parameter in the statistical analysis.

Inoculation effects on root development

The most marked effects of *Azospirillum* inoculation on plants are the various morphological changes in the root system. These changes are directly related to inoculum concentrations: higher than optimal levels had inhibitory effects, while low bacterial doses had no effect. The optimal inoculum level for seeds or seedlings of many cereals, vegetables, and industrial crop plants was 10^5-10^6 cfu/mL (Bashan 1986c; Bashan *et al.* 1989b; Kapulnik *et al.* 1985 *c*; Okon and Kapulnik 1986; Smith *et al.* 1984*b*), for corn 10^7 cfu/mL (Fallik *et al.* 1988), and for tomato in vitro >10^8 cfu/mL (Hadas and Okon 1987). However, an inoculum concentration of 10^8 - 10^{10} cfu/mL usually inhibited root development (Bashan 1986c, 1990; Barbieri *et al.* 1988; Kapulnik *et al.* 1985*c*; Morgenstern and Okon 1987*a*). However, these bacterial concentrations have not revealed how many bacterial cells per seed or seedling are required to obtain plant response.

Positive effects of inoculation have been demonstrated on various root parameters, including increase in root length, particularly of the root elongation zone (Kapulnik *et al.* 1985*b*, 1985*c*; Kolb and Martin 1985; Levanony and Bashan 1989*b*; Sarig *et al.* 1988), increase in number and length of lateral roots, which increases the root volume (Barbieri *et al.* 1986, 1988; Kolb and Martin 1985; Morgenstern and Okon 1987*a*; Tien *et al.* 1979; Venkateswarlu and Rao 1983), increases in root dry weight (Hadas and Okon 1987; Kapulnik *et al.* 1981 *a*; Morgenstern and Okon 1987*a*; Schank *et al.* 1981; Umali-Garcia *et al.* 1980), increase in the number, density, and early appearance of root hairs (Hadas and Okon 1987; Kapulnik *et al.* 1985 *c*; Martin and Glatzle l982; Morgenstern and Okon 1987 a; Umali-Garcia *et al.* 1980; Venkateswarlu and Rao 1983), increase in root surface area (Bashan 1986*c*; Fallik *et al.* 1988), enhanced cell division in the root meristem (Levanony and Bashan 1989*b*), changes in cell arrangements in the cortex (Kapulnik *et al.* 1985 *c*; Lin *et al.* 1983), and stimulation of root exudation (Heulin *et al.* 1987; Lee and Gaskins 1982). However, other studies clearly indicated a decrease in root length, mass, and volume, despite an increase in shoot growth parameters (Kucey 1988*a*; Murty and Ladha 1988; Reynders and Vlassak 1982) and no apparent change in the cell arrangement in the cortex (Levanony *et al.* 1989).

Such contradicting effects on roots by commonly available *Azospirillum* strains are apparently real since most of these morphological parameters are easily and accurately measured and were adequately analysed by reliable statistical methods. Thus, one can conclude that some other environmental factors, such as plant nutrition and irrigation, and (or) the plant genome may significantly determine the type and the magnitude of effects in any bacterial strain - plant cultivar association.

These remarkable morphological changes in the root system led to the hypothesis (Okon 1985) that improvement in plant growth and, finally, yield increase are due to a general, nonspecific improvement of the root system. A greater root volume, even without a change in its efficiency, could result in greater plant growth. Because numerous parameters are involved in this process and the nonspecific improvement of root growth has not yet been defined, this hypothesis can neither be confirmed nor disproved on the basis of the currently available data.

Root colonization by Azospirillum

Azospirillum can colonize roots externally and internally. In external colonization, the bacteria form mainly small aggregates, although many single cells may also be scattered on the root surface. These externally colonizing bacteria are embedded in the mucigel layer of the root surface (Bashan *et al.* 1986; Berg *et al.* 1979; Murty and Ladha 1987; Schank *et al.* 1979; Umali-Garcia *et al.* 1981). Both live and dead roots can be colonized (Bashan *et al.* 1986; Bashan and Levanony 1988*a*). In internal colonization, *Azospirillum* cells can colonize roots by penetrating into the root intercellular spaces (Patriquin and Döbereiner 1978; Levanony *et al.* 1989; Umali-Garcia *et al.* 1981).

Although *Azospirillum* can externally colonize the entire root system, it tends to preferentially colonize the elongation and root-hair zones (Bashan *et al.* 1986; Okon and Kapulnik 1986). In cereals, colonization is mainly on the root surface and very few bacteria are attached to the root hairs (Bashan and Levanony 1989*b*; Okon and Kapulnik 1986), whereas in rice, massive root-hair colonization was frequently observed (Murty and Ladha 1987). Penetration of *Azospirillum* into root hairs has been rarely detected and it is assumed that *Azospirillum* does not penetrate into intercellular root spaces through root hairs.

Scanning electron microscopic studies on several plant species have shown that *Azospirillum* cells are connected to the root surface and to each other within the bacterial aggregate by a network of fibrillar material (Bashan *et al.* 1986; Gafni *et al.* 1986; Hadas and Okon 1987; Okon and Kapulnik 1986; Patriquin *et al.* 1983; Umali-Garcia *et al.* 1980). The chemical nature of these fibrils is not fully defined, but there are preliminary indications that the fibrils contain proteinaceous compounds (Bashan and Levanony 1989*b*). Fibrillar attachment by the bacteria is primarily dependent on active bacterial metabolism; dead bacteria did not attach to roots, but live bacteria attached to dead plant material (Bashan *et al.* 1986; Gafni *et al.* 1986). Root surface attachment is relatively weak; slight rinsing of the roots released most of the bacteria (Bashan *et al.* 1986). Polar attachment of *Azospirillum* cells to roots was demonstrated (Patriquin *et al.* 1983; Whallon *et al.* 1985) and later confirmed (Levanony *et al.* 1989). However, detailed examination of this phenomenon revealed that it comprised only a minority of the cells. Most of the root surface was colonized by bacteria in a horizontal, thermodynamically more stable, position.

Azospirillum attachment to root surfaces occurs rapidly,

within seconds or minutes of bacterial exposure to the plant material. Most of the colonized areas on plant cells were saturated within 2 h after inoculation, varying with the bacterial growth phase and strain (Bashan and Levanony 1988*b*; Eyers *et al.* 1988*a*, 1988*b*; Gafni *et al.* 1986; Umali-Garcia *et al.* 1980). Movement of *Azospirillum* along the root surface is minimal owing to formation of multistranded fibrils. These holdfast fibrils ensure vertical bacterial transfer by the growing root tip to deeper soil layers (Bashan and Levanony 1989*a*). The possibility of bacterial movement through the mucigel without attachment to root surfaces has not yet been explored.

The specific mechanism by which *Azospirillum* attaches itself to the roots remains unknown. Several physiological, environmental, nutritional, and chemical factors enhanced or suppressed *A. brasilense* attachment to the roots (Bashan and Levanony 1989*b*; Umali-Garcia *et al.* 1980). Lectin binding has been suggested as a possible mechanism (Tabary *et al.* 1984; Umali-Garcia *et al.* 1980) and recently it was speculated that agglutinins may be located in the fibrillar material, helping cell anchorage (Bashan and Levanony 1988*b*). However, these theories have not been further explored.

Azospirillum is capable of internal colonization of the intercellular spaces of the cortex (Levanony *et al.* 1989; Patriquin and Döbereiner 1978; Whallon *et al.* 1985). Claims of vascular colonization by a specific *A. lipoferum* strain detected by light microscopy (Patriquin and Döbereiner 1978) have not been confirmed by electron microscopy when strain *A. brasilense* Cd was used (Levanony *et al.* 1989). The internal bacterial population comprised most of the total root population of *Azospirillum* in wheat (Bashan *et al.* 1986), whereas in pearl millet, most of the *Azospirillum* population was concentrated on the root surface (Matthews *et al.* 1983). Internal root colonization does not always occur. In Kallar grass, massive *Azospirillum* surface colonization was detected, but an internal population was absent (Reinhold *et al.* 1986), and in sugarcane callus, *Azospirillum* colonized only the callus surface (Berg *et al.* 1979; Vasil *et al.* 1979).

Internal and external root colonization are apparently not associated with any morphological or structural changes in the cortex cells in wheat (Levanony *et al.* 1989), although changes in the outer shape of roots in other plant species were reported (Bashan *et al.* 1989*b*; Morgenstern and Okon 1987*a*). It was further noted that inoculation may alter cell arrangement in the cortex of wheat and Setaria roots (Kapulnik *et al.* 1985 *c*; Okon *et al.* 1983). This observation was based on limited microscopic observations and should be further studied, especially since this alteration has not been confirmed by a later study using the same *A. brasilense* strain (Levanony *et al.* 1989).

The mode of *Azospirillum* penetration into the intercellular spaces is unknown. Theories suggested so far have attributed it to (*i*) bacterial invasion via disrupted cortical tissues where branches of lateral roots emerged from the main roots (Patriquin and Döbereiner 1978; Matthews *et al.* 1983; Umali-Garcia *et al.* 1980, 1981), (*ii*) invasion through lysed root hairs and mechanical injuries occurring during plant growth, and (*iii*) direct penetration through the middle lamella following pectino-lytic activity once the bacteria gain entry at splits of the epidermis-coated lateral root emergence (Umali-Garcia *et al.* 1980). Strains of this genus are known to produce some pectinases in vitro (Okon and Kapulnik 1986; Plazinski and Rolfe 1985*a*; Tien *et al.* 1981) and can inhibit the pectolytic activity of roots (Goldman and Langenbach 1987).

In conclusion, the mode of root colonization by *Azospirillum*

TABLE 1. Proposed mode of action of *Azospirillum* in plants

(A) Nitrogen fixation

Evidence for	Evidence against
Accumulation of N compounds in inoculated plants	Nif+ strains and Nif- mutants caused similar effects on plants
Bacterium has N_2-fixation ability	Positive effect on plants uneder conditions preventing N_2 fixation
Strong nitrogenase activity in inoculated plants	Negligible nitrogenase activity in responding plants
Normal plant growth after inoculation with little or no N added	Amounts of fixed N_2 are not sufficient to explain increase in plant yield
Incorporation of 5–18% of plant N from N_2 following inoculation	

(B) Plant hormones

Experimental evidence for	No experimental evidence for
Production of several plant hormones by the bacteria *in vitro*	Effect of hormone- mutants Change in hormone balance in the roots Relation between hormonal effects and production of an improved plant
Mimic of plant response towards inoculation by hormone application	
Hormone overproducing mutants caused more pronounced effect on plant growth	
Indication that inoculation changes the amount of hormones in the plant	

(C) General improvement in plant growth and mineral uptake

Experimental evidence for	No experimental evidence for
Enhancement and accumulation of minerals in plant foliage	Effect of mineral uptake - mutants
Enzymatic activities related to ion transformation in plant foliage	Enzymatic activities related to mineral and water uptake in the roots
Improvement of many growth parameters	Enhancement of mineral uptake by most strains
Improvement of water uptake	
Partial substitution of N fertilization	
Increase in proton efflux of inoculated plants	

(D) Bacterial nitrate reductase

Experimental evidence for	No experimental evidence for
The bacteria have an active enzyme	Differential changes between activities in root and foliage
NR- mutants are less effective on plants than NR+ wild type	Interaction with N_2 fixation
NR- mutants have N_2-fixation ability	
NR- mutants cause nitrate accumulation in the foliage	

may vary, depending on the bacterial strain, plant species, environmental conditions, and other unidentified factors. The interaction between all these variable creates different degrees and patterns of root colonization, different population sizes, and different colonization sites. The main colonization sites, for most plant species studied, are the elongation and root-hair zones. Root surface colonization supported by fibrillar anchoring is one of the proposed features of *Azospirillum* root colonization. Internal root colonization has been demonstrated only in a few plant species and is presumably unique to certain plant-*Azospirillum* interactions.

Proposed mode of action of Azospirillum *on plant growth*

The principal mechanism by which *Azospirillum* enhances plant growth is undetermined. However, several possible modes of action have been proposed (Table 1): N_2 fixation, which contributes N to the plant; hormonal effects, which alter plant., metabolism and growth; general improvement in the growth of the entire root system possibly related, but as yet unproven, to hormonal changes, resulting in enhanced mineral and water uptake; and bacterial nitrate reductase activity in roots, which increases nitrate accumulation in inoculated plants.

Nitrogen fixation by Azospirillum

All wild-type *Azospirillum* strains fix atmospheric nitrogen efficiently either as free-living bacteria or in association with plants and participate in several transformations in the nitrogen cycle (Heulin *et al.* 1989; Hurek *et al.* 1988; Tarrand *et al.* 1978). Following inoculation, there is an increase in the total N of shoots and grains of inoculated plants (Baldani *et al.* 1983, 1987; Boddey *et al.* 1986; Cohen *et al.* 1980; Hegazi *et al.* 1983, Kapulnik *et al.* 1981 *a*, 1983, 1985 *b*; Mertens and Hess 1984; Nur *et al.* 1980 *a*; O'Hara *et al.* 1981; Pacovsky *et al.* 1985 *b*; Rennie *et al.* 1983; Schank *et al.* 1981; Wani *et al.* 1985; Warembourg *et al.* 1987; Yahalom *et al.* 1984). Therefore, N_2 fixation was naturally the first major mechanism of action suggested for the enhancement of plant growth by *Azospirillum*. Incorporation of atmospheric nitrogen into the host plant by *Azospirillum* was evaluated mainly by the acetylene reduction assay (which simulates N_2 fixation) (Van Berkum and Bohlool 1980). However, conclusive proof that plants derive some of their N from the atmosphere came from the use of isotopic $^{15}N_2$ incorporation and ^{15}N-dilution techniques. This subject has recently been adequately reviewed (Boddey 1987; Boddey and Döbereiner 1988).

Evidence that N_2 fixation contributes to the N balance of plants is based on the common observation of an increase in the nitrogenase activity within inoculated roots (Berg *et al.* 1980; Cohen *et al.* 1980; Hegazi *et al.* 1983; Hess 1982; Kapulnik *et al.* 1981 *b*; Okon *et al.* 1983; Rao and Rajamamohan Rao 1983; Yahalom *et al.* 1984). This well-documented enzymatic activity is of sufficient magnitude to account for the increase in total N yield of inoculated plants if all the fixed N is incorporated into the plants (Sarig *et al.* 1984; Mertens and Hess 1984). Thus, on one hand, inoculation of wheat and maize has indicated that 5–10% (Kucey 1988*a*) and up to 18% (Rennie 1980; Rennie *et al.* 1983; Rennie and Thomas 1987) of the plant N was derived from N_2 fixation. In addition, inoculated plants grew normally with only a partial amount of the N fertilizer usually required for such growth (Kapulnik *et al.* 1981 b; Millet and Feldman 1986; Nur *et al.* 1980 *a*). On the other hand, studies have shown low or even negligible nitrogenase activity in plants positively responding to inoculation (Kapulnik *et al.* 1985 *a*; Lethbridge and Davidson 1983; Venkateswarlu and Rao 1983). Furthermore, of all the N fixed by the bacteria, less than 5% was incorporated into the host plants (Eskew *et al.* 1981; Okon *et al.* 1983). These amounts of fixed N are insufficient to explain total increases in N content of inoculated plants. Finally, high N fertilization levels, which inhibit N_2 fixation, did not eliminate the plant response to inoculation (Avivi and Feldman 1982; Bashan *et al.* 1989*b*, 1989*c*; Kapulnik *et al.* 1981*b*, 1982, 1983; Mertens and Hess 1984; Millet and Feldman 1986; Pal and Malik 1981; Rai and Gaur 1982; Reynders and Vlassak 1982).

The ultimate control to distinguish the contribution via N_2 fixation from other effects of bacterial inoculation is to use Nif⁻ mutants incapable of fixing N_2 but otherwise isogenic in respect to parental strains. Inoculation of cereals with Nif⁻ mutants caused the same effects as the parental strains (Barbieri *et al.* 1986; Morgenstern and Okon 1987*a*; O'Hara *et al.* 1981). Recently, tomato seedlings responded to inoculation with the site-directed Nif⁻ mutant of *A. brasilense* Cd in a manner similar to their response to the wild type (Bashan *et al.* 1989*c*). This indicates that the plant response was caused by factors other than N_2 fixation. Nevertheless, the possibility remains that N_2 fixation contributes to the plant small amounts of N,

which may be important in critical stages of plant development, such as the reproductive and the tillering stages. In conclusion, N_2 fixation occurs in many *Azospirillum* associations; the basic question in each inoculation system remains as to how much N is contributed to the plant by the bacteria and under what growth conditions, a factor that is highly variable and erratic.

Hormonal effects of Azospirillum *on plants*

Many *Azospirillum* strains produce several plant hormones in liquid culture. The major hormone produced is indole-3-acetic acid (IAA) (Barbieri *et al.* 1986; Fallik *et al.* 1989; Hartmann *et al.* 1983; Jain and Patriquin 1985; Kolb and Martin 1985; Ruckdäschel *et al.* 1988; Tien *et al.* 1979; Venkateswarlu and Rao 1983). Other hormones, detected at much lower, but biologically significant levels were indolelactic acid (Tien *et al.* 1979), indole-3-butyric acid (IBA) (Fallik *et al.* 1989), indole-3-ethanol, indole-3-methanol (Crozier *et al.* 1988), unidentified indole compounds (Hartmann *et al.* 1983), several gibberellins (Bottini *et al.* 1989; Tien *et al.* 1979), abscisic acid (ABA) (Kolb and Martin 1985), and cytokinins (Horemans *et al.* 1986; Tien *et al.* 1979).

Plant hormones affect the N_2 fixation capability of *Azospirillum* (Christiansen-Weniger 1988). Applications of external hormones, either synthetic or purified from bacterial culture, to seedlings completely reproduced the effects of *Azospirillum* on root development and morphology (Harari *et al.* 1988; Kucey 1988*b*; Tien *et al.* 1979; Zimmer and Bothe 1988). In particular, it caused changes in root length (Kolb and Martin 1985; Morgenstern and Okon 1987*a*), produced more root hairs (Kapulnik *et al.* 1985*c*; Morgenstern and Okon 1987*a*) and branching of root hairs (Jain and Patriquin 1984), produced more lateral roots (Barbieri *et al.* 1986; Tien *et al.* 1979), and enhanced the rates of cell division and differentiation in meristematic tissues (Fallik *et al.* 1989). An *Azospirillum* strain and a mutant which overproduced IAA in culture strongly affected plant root morphology (Jain and Patriquin 1985; Kolb and Martin 1985), whereas mutants that failed to produce IAA in culture had no effect on root morphology (Barbieri *et al.* 1986). Inoculation with *Azospirillum* improved the hormonal balance of a hormone-defective mutant of wheat (Inbal and Feldman 1982). Recently, higher amounts of IAA and IBA were identified in inoculated maize roots than in noninoculated plants (Fallik *et al.* 1989).

These results provide indirect evidence for the involvement of *Azospirillum* in hormonal regulation of the plant. However, before it is firmly established that hormonal effects are the principal mechanism by which *Azospirillum* promotes plant growth, the following should be investigated and supported by evidence (*i*) Do changes in root morphology have a direct effect on growth of a plant having a higher yield, i.e., these changes should not be temporary effects observed exclusively at the seedling stage. Effects on seedling root morphology may fade or disappear when the population of *Azospirillum* in the root sharply declines at mature stages of plant growth. (*ii*) Irreversible IAA-deficient mutants that are isogenic to the parental strains but are incapable of producing morphological effects on roots need to be produced. (*iii*) Changes in the hormone balance of soil-grown plants of several plant species must be clearly demonstrated. (*iv*) Since hormonal effects in plants depend on changes in equilibrium between different hormones, such changes should be demonstrated in inoculated plants.

In conclusion, the study of the hormonal effects of *Azospiril-*

lum is a promising avenue, which should be further explored. However, more direct, rather than circumstantial, evidence is needed before the hypothesis is accepted.

Improvement of root development, mineral uptake, and plant-water relationships by Azospirillum

In addition to increasing (Kapulnik *et al.* 1981 *a*, 1985 *c*) or decreasing (Kucey 1988 *a*) many root parameters, plant inoculation with *Azospirillum* affected many foliage parameters. These changes were directly attributed to positive bacterial effects on mineral uptake by the plant. Enhancement in uptake of NO_3^-, NH_4^+, PO_4^{2-} K^+, Rb^+, and Fe^{2+} by *Azospirillum* (Barton *et al.* 1986; Jain and Patriquin 1984; Kapulnik *et al.* 1985*b*; Morgenstern and Okon 1987*b*; Murty and Ladha 1988; Lin *et al.* 1983; Sarig *et al.* 1988) was proposed to cause an increase in foliar dry matter and accumulation of minerals in stems and leaves. During the plant reproductive period, these minerals could have been transferred to the panicles and spikes and finally resulted in a higher yield.

Increased mineral uptake by plants has been suggested to be due to a general increase in the volume of the root system and not to any specific enhancement of the normal ion uptake mechanism (Morgenstern and Okon 1987*b*; Murty and Ladha 1988). It has been further suggested that *Azospirillum* inoculation may promote availability of ions in the soil by helping the plant scavenge limiting nutrients (Lin *et al.* 1983); this may explain accumulation of N compounds in the plant without any apparent N_2 fixation. The plant may take up N more efficiently from the limited supply in the soil, resulting in a lower requirement of N fertilization to attain a certain yield. Supporting evidence for increased mineral uptake by inoculated roots is provided by enhancement in proton efflux activity of wheat roots inoculated with *Azospirillum* (Bashan 1990; Bashan *et al.* 1989*a*). It is well known that proton efflux activity is directly related to the balance of ions in plant roots.

In addition to improved mineral uptake, *Azospirillum* inoculation improved water status in stressed sorghum plants. Inoculated plants were less water stressed, having more water in their foliage, higher leaf water potential, and lower canopy temperature than noninoculated plants. Total extraction of soil moisture by *Azospirillum*-inoculated plants was greater and water was extracted from deeper layers in the soil profile. Therefore, sorghum yield increase in inoculated plants was attributed primarily to improved utilization of soil moisture (Sarig *et al.* 1988).

It is likely that improved mineral and water uptake play a vital role in *Azospirillum*-plant association. However, the descriptive data presented so far have not shown whether these improvements are the cause or the result of other mechanisms such as changes in plant hormonal balance. Furthermore, the wide range of enzymatic activities related to these phenomena were poorly studied and no apparent evaluation of *Azospirillum* mutants deficient in induction of mineral and water uptake by plants has been made. Finally, it should be emphasized that very few strains have been evaluated and it is doubtful if all *Azospirillum* strains possess these abilities. There is evidence that some *A. brasilense* strains failed to improve uptake of several ions but nevertheless improved plant growth (Bashan *et al.* 1990).

Azospirillum *nitrate reductase in plants*

An alternative to N_2 fixation as an explanation for N accumulation following *Azospirillum* inoculation of wheat plants only is the bacterial nitrate reductase (NR) theory. NR activity of wheat leaves was decreased by inoculation with some *Azospirillum* strains. Inoculation of plants with NR$^-$ mutants resulted in minimal plant response concomitant with an increase in leaf NR compared with inoculation with the parental NR$^+$ strain (Ferreira *et al.* 1987). Inoculation of field-grown plants with *A. brasilense* Sp-245 and its NR$^-$ mutant confirmed that the mutant was significantly less effective in increasing yield than the parental strain (Boddey and Döbereiner 1988). These phenomena indicate that the effect of some *Azospirillum* strains on plants is not solely via N_2 fixation (both the parental and the mutant strains have this ability) but rather due to an increase in nitrate assimilation. The parental strain aided nitrate reduction in the roots and thus decreased nitrate translocation to the leaves, while inoculation with the NR$^-$ mutant caused direct translocation and reduction of nitrate in the plant foliage.

This theory might partially explain the observation of increased N accumulation in shoots because the unaffected N_2-fixation ability may also contribute N to the plants in addition to NR activity.

Specificity and variability in Azospirillum

One of the most controversial questions concerning the *Azospirillum* association is that of plant-strain specificity as it affects plant growth. Specific differences between responses of C_3 and C_4 plants was suggested; *A. lipoferum* was the predominant species colonizing C_4 plants and *A. brasilense* was the predominant species associated with C_3 plants in tropical zones (Baldani and Döbereiner 1980; Baldani *et al.* 1986). Similar host plant preference was found in temperate zones (Haahtela *et al.* 1981; Lamm and Neyra 1981). When the bacterial species was inoculated onto the respective plant species, success was more frequent when the proper plant bacterial species combination was used (Baldani *et al.* 1983, 1987; Pereira *et al.* 1988; Reynders and Vlassak 1982). Different morphogenetic effects on wheat root hairs have been attributed to different strains (Jain and Patriquin 1984; Patriquin *et al.* 1983). Specificity can occur at the plant cultivar level; only a few of many tested plant cultivars responded to inoculation with a given strain of *Azospirillum* (Bouton *et al.* 1979; Millet *et al.* 1986; Wani *et al.* 1985). On the other hand, studies with *A. brasilense* Cd/Sp7, the type strain for *A. brasilense* commonly used as a reference, indicated no plant specificity. This strain positively affected many winter and summer cereals, vegetables, and industrial crop plants (Bashan *et al.* 1989*b*; Kapulnik *et al.* 1981 *c*, 1983; Smith *et al.* 1984*b*; Yahalom *et al.* 1984).

The main difficulties in assessing *Azospirillum* specificity are (*i*) lack of comparison tests for strains obtained from different sources on certain host plants and (*ii*) the limited number of strains tested thus far. Furthermore, there are indications that specificity is not at the bacterial species level but rather at the strain level. A quick screening test to assess plant - bacterial strain association is essential to predict successful plant-bacteria combinations prior to testing in the field.

In conclusion, like *Rhizobium strain* selection, the importance of *Azospirillum* strain selection is a crucial consideration in determining specificity. Numerous strains must be isolated from different origins, characterized, and tested under various environmental conditions for each plant species.

Interaction of Azospirillum *with other soil-rhizosphere micro-flora*

After application to the soil, *Azospirillum* cells must adapt quickly to the ever-changing conditions of the plant rhizo-

sphere, including frequent changes in nutrient availability and interaction with indigenous microorganisms competing for those nutrients. These interactions could be antagonistic or synergistic or a predator-prey type, where *Azospirillum* cells possibly serve as available prey for the always nutrient-deficient micro- and macro-fauna.

The most studied microbial interaction of *Azospirillum* involves *Rhizobium*. On one hand, greenhouse and field trials have shown that a simultaneous inoculation of *Azospirillum* and *Rhizobium or* addition of *Azospirillum* to naturally rhizobia colonized legumes resulted in increased N$_2$ fixation, greater number of nodules, and eventually a yield increase (Iruthayathas *et al.* 1983; Rai 1983; Sarig *et al.* 1986). On the other hand, laboratory studies of mixed inoculation resulted in conflicting data; *A. brasilense* applied before *Rhizobium* increased nodule formation in the non-root-hair zone, even when *Rhizobium* was applied at sub-nodule-formation concentration. This increased susceptibility of legumes to *Rhizobium* infection was attributed to a stimulating effect of hormones excreted by *Azospirillum*. These hormones induced formation of a larger number of epidermal cells that differentiated into root hairs, favored for primary infections by *Rhizobium* (Schmidt *et al.* 1988; Yahalom *et al.* 1987). However, negative effects of mixed inoculation that prevent clover nodulation occurred especially when *Azospirillum* cells outnumbered *Rhizobium* cells in the mixture (Plazinski and Rolfe 1985*b*, 1985*c*, 1985*d*). Apparently, root-hair colonization by *Azospirillum* blocked infection sites of *Rhizobium*. These contradicting reports indicate that the exact interaction between *Azospirillum* and *Rhizobium* should be first defined before any conclusions can be drawn.

In contrast to the unclear relations that prevail between *Azospirillum* and *Rhizobium, synergistic* effects of mixed inoculation of *Azospirillum* and vesicular-arbuscular (VA) mycorrhizal fungi resulted in significant increase in growth and P content of plants. This dual inoculation could completely replace application of N and P fertilizers (Barea *et al.* 1983; Pacovsky 1988; Pacovsky *et al.* 1985 *a*; Subba Rao *et al.* 1985a, 1985b) and enhance infection of plants by mycorrhizal fungi (Barea *et al.* 1983). However, the shortage of data obtained thus far can only encourage further research on this interaction since the basic nature of the interaction among bacteria, fungi, and plant has yet to be described.

A different synergism resulting from *Azospirillum* inoculation is enhancement of straw decomposition by cellulolytic bacteria. Mixed bacterial cultures either in liquid culture or in soil were more efficient in decomposing straw than cellulolytic bacteria alone. The microbial interaction also enhanced N$_2$ fixation by *Azospirillum*. Cells of both species were found in close proximity in the straw, facilitating the mutually beneficial association (Halsall and Gibson 1985, 1986; Halsall and Goodchild 1986; Halsall *et al.* 1985; Markus and Kramer 1988). Such synergism could provide an indirect explanation for the highest known yield increase by *Azospirillum*, which occurred in soil amended with straw (Hegazi 1988; Hegazi *et al.* 1983). Nevertheless, similar to *Azospirillum* interaction with VA mycorrhizal fungi, this interaction should be first defined in order to evaluate its potential.

The above interactions represent only a minute fraction of *Azospirillum* relationships with other rhizosphere microorganisms. The interactions with common, indigenous, predominant species of pseudomonads, azospirilla, and Bacillus populations (10^6-10^9 cfu/g) have been scarcely studied. *Azospirillum* failed to serve as a biocontrol agent (Hadas and Okon 1987) and

application of Pseudomonas sp., which outnumbered *Azospirillum*, inhibited its beneficial effect on plants (Fallik *et al.* 1988). Many azospirilla produced bacteriocins, which surprisingly also inhibited other *Azospirillum* species (Oliviera and Drozdowicz 1981, 1987; Skorupska *et al.* 1985). These unidentified bacteriocins disappeared after application to several soils but persisted in a soil low in organic matter and clay minerals (Oliveira and Drozdowicz 1988). The ecological significance of *Azospirillum* bacteriocins is unknown.

In vitro antagonistic effects on *Azospirillum* by some soil microorganisms, especially streptomycetes and fungi, have been shown (Drozdowicz and Ferreira Santos 1987; Kulinska and Drozdowicz 1983; Zuberer and Roch 1982). Many strains of *Azospirillum* are highly resistant to a wide range of antibiotics (Bashan and Levanony 1985; Baldani *et al.* 1986; Döbereiner and Baldani 1979; Horemans *et al.* 1987) and isolation of antibiotic-resistant *Azospirillum* strains is a simple task. Nevertheless, the effect of antibiotic-producing microorganisms on *Azospirillum* in the soil has not been studied. Taking advantage of the natural antibiotic resistance of *Azospirillum*, improvement of root colonization by *Azospirillum* and subsequent plant yield increase in wheat plants grown in pots was obtained by temporarily depressing the competing natural microflora, using inhibiting substances to which *Azospirillum* is resistant. Although this inhibition lasted only for a few weeks, it gave a significant advantage to *Azospirillum* cells in root colonization (Bashan 1986*a*). Obviously, this practice cannot be directly adapted to the field as a vast amount of soil, naturally occupied by the plant root system, would have to be treated, a practice which would entail soil pollution and high cost. However, this approach should be further evaluated using cheap licence agrochemicals.

The size of *Azospirillum* populations has been estimated as 1-10% of the total rhizosphere population (Okon 1985). Routine counts of *Azospirillum* in the rhizosphere indicate that this evaluation is an overestimate. In plants growing in soil, *Azospirillum* populations are relatively small; the highest population was recorded in summer cereals in Brazil (10^6-10^8 cfu/g (Baldani *et al.* 1983)). Usually *Azospirillum* populations are much smaller, reaching an average population size of 10^3-10^6 cfu/g in wheat (Balandreau 1986; Bastian and Wolowelsky 1987; Bastian *et al.* 1987; Negi *et al.* 1987). On several occasions an even smaller rhizosphere population was reported (Albrecht *et al.* 1983; Harris *et al.* 1989; Smith *et al.* 1984*b*). Therefore, a more accurate estimation of *Azospirillum* populations should be within the range of 0.001-1 % of the total rhizosphere population (O'Hara *et al.* 1981).

There are a relatively large number of studies related to the microbial interactions of *Azospirillum*. However, the data are highly fragmented, spread on numerous different aspects, and are of a descriptive nature. All these factors make it unreliable to predict the most important interactions. Because *Azospirillum* accounts for only a small fraction of the rhizosphere population, many other bacterial species (Balandreau 1986) should affect *Azospirillum* in the rhizosphere and should be considered when *Azospirillum* is applied to soil.

Azospirillum as a competitor in the rhizosphere.

A current analysis of the intensive studies on physiological and biochemical properties of *Azospirillum* revealed that this genus has no unique or distinguishing feature compared with other rhizosphere bacterial genera (Pedrosa 1988); yet single species or even individual strains of *Azospirillum* possess

characteristics that are normally found only in several different bacterial species. This creates an organism that is capable of successfully competing in many plant species rhizospheres despite the overwhelming population of indigenous rhizosphere microflora and that can colonize plant roots, under field conditions, from an extremely low bacterial inoculum level (10^6 cfu/m^2).

By definition, all wild-type *Azospirillum* are N$_2$ fixers, which can fully support their N requirements (Tarrand *et al.* 1978). Since their nitrogenase is not protected from oxygen, they must fix nitrogen under microaerophilic conditions (Hartmann and Hurek 1988) and some strains exhibit strong aerotactic response (Barak *et al.* 1982*a*, 1982*b*; Das and Mishra 1984; Del Gallo *et al.* 1988; Hurek *et al.* 1987; Okon *et al.* 1980; Reiner and Okon 1986). *Azospirillum* is nutritionally versatile. Many alternative metabolic pathways allow it to consume a wide variety of organic acids, sugars, and amino acids available in the rhizosphere from plant and microbial sources (Okon 1982). Nearly all strains are highly motile, both in vitro and in the soil, and they possess significant chemotactic ability towards an array of compounds (Barak *et al.* 1983; Bashan 1986d; Bashan and Levanony 1987; Heinrich and Hess 1985; Mandimba *et al.* 1986; Okon *et al.* 1980; Reinhold *et al.* 1985, 1988b). In normal growth, *Azospirillum* accumulates large amounts of the storage material poly-β-hydroxybutyrate, which can be stored for use later or during long-term survival (Berg *et al.* 1979; Levanony *et al.* 1989; Tal and Okon 1985). Under stress conditions the bacteria are capable of cyst and floc or aggregate formation, both of which significantly improve survival (Bleakley *et al.* 1988; Lamm and Neyra 1981; Madi *et al.* 1988; Murray and Moyles 1987; Sadasivan and Neyra 1985, 1987). Two species, *A. amazonense* and *A. halopraeferans*, have resistance to acids, or to salt and high temperatures (Hartmann 1988; Reinhold *et al.* 1988*a*), which improves *Azospirillum* survival in the rhizosphere. Unlike the limited survival capacity of *Azospirillum* in the soil, all studies on *Azospirillum* survival in its natural microecological niche concluded that it survives for prolonged periods of time. Only the size of the population varied. *Azospirillum* strains survived in roots during the winter season under temperate conditions but at low levels (De Coninck *et al.* 1988; Harris *et al.* 1989; Horemans *et al.* 1988). Survival in roots was shown during the entire growth season of cereals (Bashan *et al.* 1987; Bashan and Levanony 1987; Jagnow 1982) and rice (Nayak *et al.* 1986). Inability of *Azospirillum* populations to recover to original size after winter has been shown only under temperate conditions (Harris *et al.* 1989; Horemans *et al.* 1988). The ecological importance of this long-term survival ability has yet to be determined; whether *Azospirillum* can reestablish its population to the extent that it can colonize the next season's crop is not known. Long-term survival and reinoculation ability are obvious obstacles to commercial exploitation of *Azospirillum* technology.

In contrast to some superior features, *Azospirillum* is parasitically colonized by *Bdellovibrio* sp. in soil (Germida 1987) and serves as a prey for soil protozoa. In conclusion, *Azospirillum* has a potential as a successful competitor as well as a good survivor in the rhizosphere. The various phases of *Azospirillum* in the rhizosphere are shown in Fig. 1.

Interaction of Azospirillum *with soil particles*

Inoculation of plants with *Azospirillum* is usually done by applying the bacteria to the soil near the germinating seedling (Bashan 1986*b*; Okon and Hadar 1987). During this process

the bacterium is exposed to natural physical forces and interactions that prevail between soil bacteria and soil particles. To overcome these barriers and colonize plant roots, *Azospirillum* cells must create a substantial physical force to allow movement through the soil (Bashan 1986*d*; Bashan and Levanony 1987). Few studies have addressed the interaction of *Azospirillum* with soil particles. *Azospirillum* cells were usually irreversibly adsorbed by the upper fraction of the soil profile in a charge-charge interaction mainly with clays and organic matter. Soil physical and chemical conditions such as pH, flooding, dry regime, and availability of bacterial chemoattractants greatly affect adsorption of *Azospirillum* to soil to different extents (Bashan and Levanony 1988*c*; Horemans *et al.* 1988). Attachment of *Azospirillum* to pure sand, which lacked clays and organic matter, was weak and accomplished by a network of protein bridges produced between the bacteria cell and the quartz particles. Protein-bridge formation was mainly controlled by nutrient availability (Bashan and Levanony 1988d).

Most *Azospirillum* strains are rhizosphere bacteria; however, numerous strains occur in the soil. *Azospirillum* was originally isolated from sandy soil (Beijerinck 1925); however, bacteria resembling *Azospirillum* occur only in alluvial and not in sandy soils in Somali (Favilli *et al.* 1988). Low levels of *Azospirillum* persist in many Belgian fields regardless of soil texture (De Coninck *et al.* 1988; Horemans *et al.* 1988), whereas high indigenous levels of *Azospirillum* are common in tropical soils (Döbereiner 1988; Döbereiner *et al.* 1976; Patriquin *et al.* 1983). *Azospirillum* inocula survived poorly in soil in the absence of plants both in the field and in the greenhouse, and almost disappeared after less than 15 days (Albrecht *et al.* 1983; Bashan and Levanony 1987, 1988*c*; Horemans et ad. 1988; Smith *et al.* 1984*b*). Nevertheless, a fraction of the population survived in the soil over prolonged periods if stress conditions did not pervail (Hegazi 1988; Gemida 1986; Jagnow 1982; Sadisavan et ad. 1986*a*; Schank *et al* 1985).

In conclusion, although *Azospirillum* is a typical rhizosphere bacterium and no important bacterial growth phase in the soil per se has yet been found, it shares some of its soil adsorption features with other common soil bacteria. Despite its instant adsorption to soil particles upon inoculation, *Azospirillum* can overcome it by its self-motility through the soil to its target plant (Bashan 1986d).

Genetics and immunology of Azospirillum

Genetic analysis of *Azospirillum* has been restricted mainly to the nitrogenase structural genes (*nifHDK*) (Bazzicalupo *et al.* 1987; Bozouklian and Elmerich 1987; Fischer *et al.* 1986; Singh and Klingmüller 1986). These genes are the only *nif* genes that show homology to the *Klebsiella pneumoniae nif* genes. This homology facilitated cloning and characterization of these genes (Fahsold *et al.* 1985; Jara *et al.* 1983; Quiviger *et al.* 1982; Singh and Klingmüller 1985). On the other hand, very little is known about *Azospirillum* genetics as related to its association with plants (Elmerich 1984). The main difficulties in *Azospirillum* genetic analysis are shortage of *Azospirillum* mutants, together with the lack of an indigenous genetic recombination system (conjugation or transduction). To overcome this latter barrier, many studies have used the advantage that *Azospirillum* harbors large cryptic plasmids (Pampaluna *et al.* 1988; Skorupska *et al.* 1985). Although there is no uniformity between plasmid patterns in different strains (Elmerich and Franche 1982; Fani *et al.* 1986; Singh and Wenzel 1982), these plasmids are assumed to encode function for plant-bacterial associations like those in *Rhizobium*.
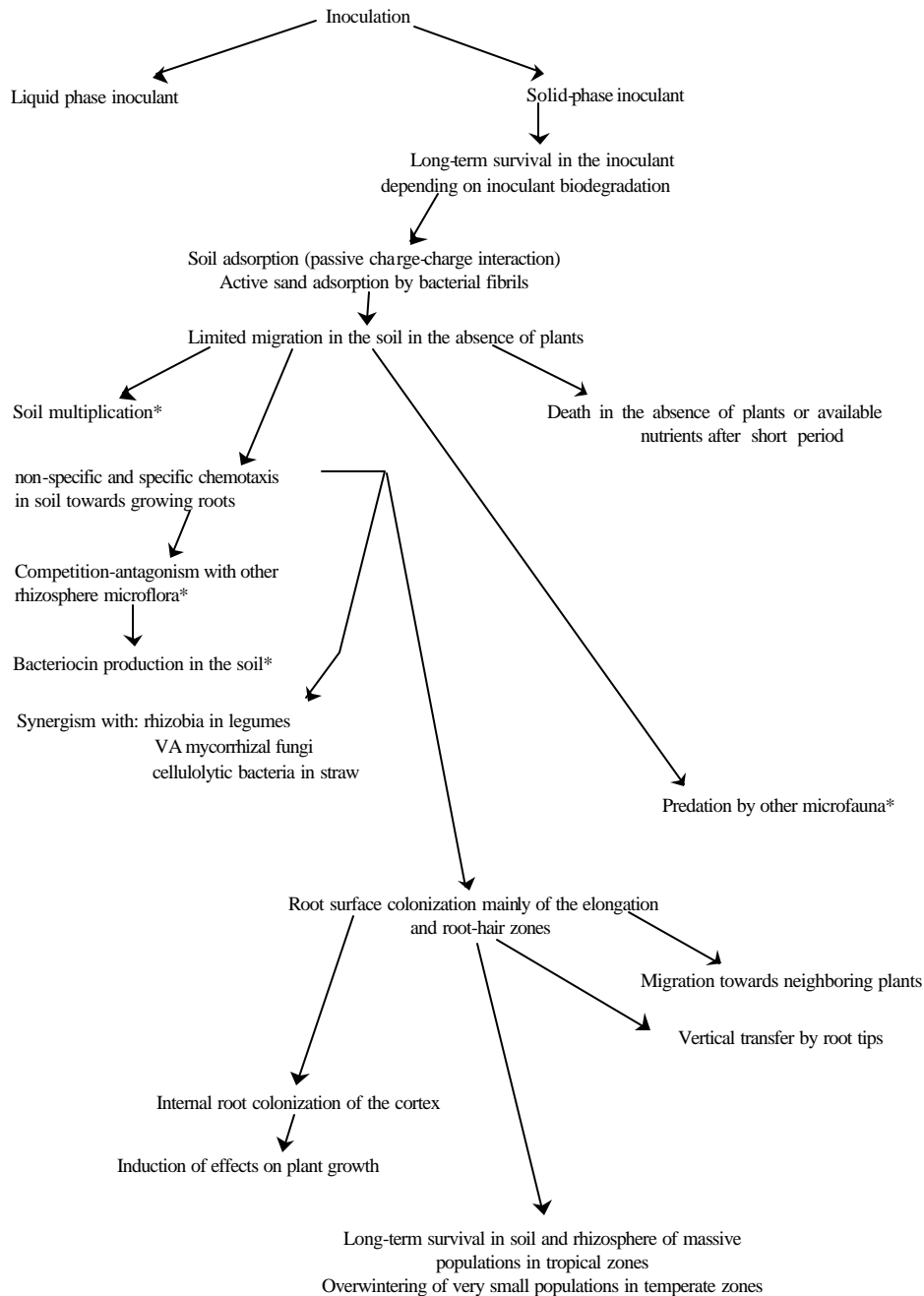
FIG. 1. Phases of *Azospirillum* in the rhizosphere.*, indications only.

An alternative approach to study the association was use of site-directed transposon mutagensis (Abdel-Salam and Klingmüller 1987; El-Khawas and Klingmüller 1988; Singh 1982; Singh and Klingmüller 1986; Vanstoekem *et al.* 1987, 1988). Many Nifmutants of *Azospirillum* have been isolated by different methods (Barbieri *et al.* 1986; de Araujo *et al.* 1988; Jara *et al.* 1983; Pedrosa and Yates 1984; Singh and Klingmüller 1986, 1988; Vanstoekem *et al.* 1987). Nif⁻ mutants induced by site-directed mutagenesis can yield conclusive results regarding nitrogenase participation in *Azospirillum* associations. As a result of the *nif* deletion such mutants cannot revert. Recently, such a Nif⁻ deletion mutant of *A. brasilense*

Cd has been shown to have positive effects on tomato seedlings similar to those of the wild-type strain, indicating no contribution of nitrogenase activity to this association (Bashan *et al.* 1989c).

Different genetic techniques showed homology to *nod* (nodulation) and *hsn* (specificity) of *Rhizobium* and to *Agrobacterium* chromosomal virulence genes in *Azospirillum*. However, it is premature to conclude that these genes are functioning in *Azospirillum* (Elmerich and Franche 1982; Elmerich *et al.* 1987; Fogher *et al.* 1985; Galimand *et al.* 1988; Piana *et al.* 1988; Waelkens *et al.* 1987). IAA-overproducing and IAA-deficient mutants have been isolated (Abdel-Salam and Kling-

müller 1988; Barbieri *et al.* 1986, 1988; Hartmann *et al.* 1983), but their role in the plant-bacteria association has not yet been determined.

In conclusion, transposon mutagenesis has been shown to be a powerful tool in genetic analysis of *Azospirillum* since it enables the generation of large number of transposon-tagged mutants differing in different genes. However, genetic understanding of *Azospirillum* is not yet sufficient to effect major improvement in *Azospirillum* inoculation technology.

Immunological study of *Azospirillum* is one of the most neglected areas in *Azospirillum* research. A few studies have been conducted using polyclonal antibodies. Very little is known about *Azospirillum* antigens. Specific antigens for *A. brasilense* Cd were found in its exopolysaccharide and flagella (Levanony and Bashan 1989*a*), and different antigens were found in the different flagella types (Hall and Kreig 1984). Different species of this genus share common antigens and, therefore, extensive cross-reaction occurs among many strains (Dazzo and Milam 1976; De-Polli *et al.* 1980; Kosslak and Bohlool 1983).

These studies reveal that specific polyclonal antibodies can be successfully used in rhizosphere studies for detection and enumeration of *Azospirillum*. *Azospirillum* cells were qualitatively identified in the rhizosphere by a fluorescent antibody technique (Schank *et al.* 1979), peroxidase-antiperoxidase labelling (Matthews *et al.* 1983), and by several other immunotechniques (Ladha *et al.* 1982). *Azospirillum brasilense* Cd has been specifically quantified in the rhizosphere of several cereals by employing indirect and competition enzyme-linked immunosorbent assays (ELISA; Levanony *et al.* 1987) and by an assay using avidin-biotin complex incorporated into the ELISA procedures (Levanony and Bashan 1990). This strain was further identified in the cortex of wheat roots, using an immuno-gold labelling technique (Levanony *et al.* 1989). The accuracy and usefulness of the immuno-techniques proved to be an essential practical tool in rhizosphere research despite the lack of basic immunological knowledge about *Azospirillum*.

Agrotechnical aspects: inoculants and interaction with pesticides

An important objective of understanding the *Azospirillum* system is commercial application of the bacteria in agricultural systems of both modern and developing countries. Astonishingly, very little has been published concerning the agrotechnical aspects of this system, i.e., potential effects of inoculation with *Azospirillum* in conjunction with different chemical compounds applied in a commercial field. Presumably, this type of data, which was expected to be developed by research and development companies, has not been published.

Even after establishing the best *Azospirillum*-plant combination, the problems of successful application in commercial crop production still prevail. Bacteria should reach the root even if the root system is widely spread; bacterial inoculation should be at the precise time needed by the plant (Bashan 1986*c*); inoculation techniques should be practical, economical, and easy to accomplish for the farmer; the formulated product should deliver sufficient inoculum to the plant, must be competitive with existing commercial standards, and must possess a long shelf life.

To date, only a few different methods are used to inoculate with *Azospirillum*. The simplest inoculation method is by application of bacteria in liquid suspension either directly to the soil or to the seeds. This technique was used in numerous greenhouse and field experiments (Albrecht *et al.* 1981; Fallik *et al.* 1988; Millet and Feldman 1986; Reynders and Vlassak 1982; Smith *et al.* 1984) but is inadequate because *Azospirillum* survives poorly in soil in the absence of a carrier. More reliable procedures use various organic carriers (Okon 1985; Sadasivam *et al.* 1986*b*). The best yield results have been obtained from peat suspensions dripped into the sowing furrow or by spreading granular peat inoculant at the time of sowing (Okon and Hadar 1987). These practical inoculants cannot provide some of the requirements of a good inoculant owing to uncontrolled bacterial release and several technical difficulties (Bashan 1986*b*), and they thus probably result in inconsistent yield results. A different approach using encapsulated freeze-dried bacteria in dry alginate beads overcomes some of the problems of liquid and peat inoculants and fulfills many of the requirements for a good practical inoculant. It is dry, synthetic, simple to use, uniform, biodegradable by soil microorganisms, and nontoxic in nature, contains a large uniform bacterial population, provides for the slow release of the bacteria for long periods, and may be produced on a large scale (Bashan 1986*b*; Bashan *et al.* 1987). Development of advanced inoculants is a most important task in future application of *Azospirillum*.

Agrochemicals, especially pesticides, may exhibit undesirable side effects on nontarget microorganisms in the environment. Research in this area has been largely neglected. The only information available is from experiments conducted *in* vitro where herbicides had some effect on nitrogenase activity (Haahtela *et al.* 1988; Jagnow *et al.* 1979) and on *Azospirillum* growth (Gadkari 1987, 1988). Incorporation of insecticides into the growth medium caused either cell disruption of *Azospirillum* and formation of cyst-like bacteria (Mano *et al.* 1988) or increase in N_2 fixation and IAA production (Jena *et al.* 1987). Applied research in *Azospirillum* technology must focus on this area.

Concluding remarks and future prospects

Azospirillum was initially tested for agronomic exploitation more than a decade ago as a result of two basic features: (*i*) its ability to fix atmospheric N and (*ii*) its intimate association with roots of cereals and grasses. Although no special morphological structure was ever found in or on inoculated roots, increase in N content of inoculated plants as well as reported yield increases ranging from 10 to 200% above noninoculated plants defined *Azospirillum*-cereal interaction as equivalent to *Rhizobium-legume* association. This prompted numerous field inoculation attempts, mostly performed between 1978 and 1984. The large amount of data from these worldwide field experiments, combined with laboratory and greenhouse experiments on the bacterial mode of action, led to several surprises. Despite the optimistic initial results, *Azospirillum* inoculation in the field proved to be inconsistent and unpredictable; experimental results were difficult to repeat even if experiments were performed in an identical fashion. Initial estimates that 50-70% of all field experiments worldwide were successful are probably an overestimation since many failures were never reported, especially when they were performed by commercial companies. These inconsistencies led to a great reduction in field expementation with *Azospirillum*. Very few field studies are published annually. Current estimations of future success, optimistic at the present level of knowledge, are that inoculation of crops with *Azospirillum* can result in an average 10-15% yield increase in fertilized areas and up to 20% under less-developed agricultural practices, or semi-arid conditions resem-

bling Israeli soils and climate (Okon 1985; Okon and Hadar 1987), with possibly a high percentage of inconsistency.

Complementary to the inconsistency in yield results, N_2 fixation in general was regarded to be of little significance concerning the contribution of *Azospirillum* to plant growth. Despite a few studies consistently demonstrating a direct significant contribution from N_2 fixation, most studies, carried out worldwide under diverse climatic and soil conditions, showed only scarce assimilation, if any, of the N fixed by *Azospirillum* to the plant. Recent experiments that employed Nif$^-$ *Azospirillum* showed beneficial effects on plant growth similar to the wild-type strain (Nif$^+$), indicating an alternative mechanism(s) involved in this association.

Several mechanisms of plant-microbe interaction may participate in the association and affect plant growth, including N_2 fixation, hormonal effects, general improvement in root growth resulting in improved mineral and water uptake, and activity of bacterial nitrate reductase in the root system. However, there are no quantitative data to support the notion that one of these mechanisms can be solely responsible for induction of such changes in the plant. Therefore, an additive hypothesis is more appropriate to describe the effect of *Azospirillum* on plants: probably more than one mechanism participates in the association. They operate simultaneously or in succession. The sum of their activities, when induced under the proper environmental conditions, results in the observed changes in plant growth. This hypothesis may also explain the frequent failure of inoculation to yield consistent results. Presumably one or more mechanisms are inactive or only partially active, thus maximal benefit expected from the association is rarely achieved. This hypothesis, therefore, may define *Azospirillum* as "plant growth promoting rhizobacteria" (PGPR) and not as previously defined, an "associative nitrogen fixer."

Genetic analysis of *Azospirillum* is lacking for nearly all the genes relevant to plant-bacteria interaction and to bacterial functions as well, except for the nitrogenase structural genes. Therefore, it is premature to foresee in the near future a major genetic manipulation that will create genetically engineered *Azospirillum* with superb capabilities in the rhizosphere. Thus, the conventional approach of screening plant-strain interaction may be a more useful tool. Although many strains of *Azospirillum* have been isolated worldwide and stored, only a few are comprehensively described. Very few field-tested isolates of *Azospirillum* are available and it is far behind strain availability of *Rhizobium*. Since *Azospirillum* is widely distributed, exploitation of yet undiscovered strains may be a useful source of new and more beneficial associations.

The ultimate test for even the most beneficial isolate is its ability to survive and to colonize plant roots successfully in the presence of larger numbers of other indigenous rhizosphere microorganisms. The study of interactions between *Azospirillum* and its natural competitors is in its infancy. This obstacle must be overcome, along with development of more effective inoculation methods, before *Azospirillum* can be used reliably as a beneficial inoculant for agriculture.

In conclusion, despite the vast amount of data accumulated on *Azospirillum* during the last 15 years, our knowledge of this association system is too limited to ensure a consistent positive interaction between the bacteria and the plant, let alone commercial exploitation of *Azospirillum* technology. It is suggested that the direction in which *Azospirillum* research should proceed, to gain the full potential of this association, is towards more basic understanding of the underlying funda-

mental components of the system and less towards full-scale field experiments. We assume that this approach will be the best in ultimately harnessing *Azospirillum* activity for the benefit of mankind.

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CAN. J. MICROBIO L. VOL. 36, 1990

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