

## AZOSPIRILLUM: PHYSIOLOGICAL PROPERTIES, MODE OF ASSOCIATION WITH ROOTS AND ITS APPLICATION FOR THE BENEFIT OF CEREAL AND FORAGE GRASS CROPS

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The free-living nitrogen-fixing bacterium *Azospirillum* species formerly known as *Spirillum lipoferum* (Beijerinck) has been described in detail by Tarrand et al., 1978. Based on DNA homology studies, two species of the genus have been characterized: *Azospirillum brasilense* and *A. lipoferum*. DNA composition of the genus is 69-71% G + C. The colonies are pink-red, drying out and turning rough with folded, wavy surfaces. Cells of *Azospirillum* are highly motile, half curved (vibroid), gram negative rods, having a diameter of 1.0  $\mu\text{m}$ , with a polar flagellum when grown on liquid medium. Lateral flagella with shorter wavelengths could be observed in *A. brasilense* growing in agar medium. Cells contain prominent intracellular granules of poly- $\beta$ -hydroxybutyrate.

### *Physiological Properties*

The physiological and biochemical properties of *Azospirillum* have recently been reviewed (Neyra & Döbereiner, 1977; Burris et al., 1978; van Berkum & Bohlool, 1980; Döbereiner & De-Polli, 1980).

The nitrogenase complex of *A. brasilense* sp. 7 is composed of the normal Mo-Fe and Fe proteins, but it also possesses the activating factor for the Fe protein (Ludden et al., 1978).

*A. brasilense* is not capable of using glucose as a sole carbon source for growth or nitrogen fixation, as it lacks the ability to ferment sugars. Vitamins are not required for growth. *A. lipoferum* differs from *A. brasilense* in its ability to use glucose, its requirement for biotin and the formation of wider, longer s-shaped or helical cells.

Both *A. brasilense* and *A. lipoferum* oxidize organic acids and grow very well on them. Galactose and arabinose are also utilized by both species.

Many azospirilla are capable of reducing  $\text{NO}_3^-$  under anaerobic conditions (Neyra & Döbereiner, 1977). They are preferentially microaerophilic organisms, i.e. they actively seek microaerobic conditions (low  $\text{pO}_2$ ), and develop a growth pellicle below the surface in semi-solid medium when grown both under  $\text{N}_2$  or combined nitrogen sources. Also, azospirilla are chemotactically attracted to oxygen (aerotaxis) and to

organic acids, amino acids and some sugars (Okon et al., 1980; Barak et al., 1982).

Nitrogen fixation-takes place under very low  $pO_2$  (0.005–0.007 atm), its nitrogenase being poorly protected from oxygen damage (Okon et al., 1976a, 1977b; Nelson & Knowles, 1978). However, when the cultures are provided with combined nitrogen growth of the organisms is typically aerobic (Okon et al., 1976a).

As shown in continuous culture studies (Nur et al., 1982), azospirilla are very versatile organisms. They are readily adaptable to different dilution rates in the chemostat and to different  $pO_2$ . At high  $pO_2$  (0.16–0.18 atm) they develop high succinic oxidase and superoxide dismutase activities (Nur et al., 1982) under micro-aerobic conditions. They dispose of excess reductant by synthesis of poly- $\beta$ -hydroxybutyrate (Okon et al., 1976b; Nur et al., 1982), and under anaerobic conditions, they use  $NO_3^-$  as final acceptor of electrons (Nelson & Knowles, 1978).

Some strains of *A. brasilense* such as strain Cd produce carotenoids under increasing  $pO_2$  and are capable of fixing nitrogen at slightly higher  $pO_2$  as compared to non-pigmented strains (Nur et al., 1981).

It has recently been demonstrated that the usually heterotrophic *Azospirillum*, as well as *Azotobacter* and *Rhizobium*, are capable of autotrophic growth on  $H_2$  and  $CO_2$  as sole energy and carbon sources, respectively (Yates et al., 1981). *Azospirillum* possesses an uptake hydrogenase, capable of utilizing the hydrogen evolved from the nitrogenase reaction (Chan et al., 1980).

Azospirilla can carry out most of the nitrogen transforming reactions which occur in the soil: nitrogen fixation, assimilation of  $NH_4^+$  and  $NO_3^-$ , mineralization of organic-N and denitrification. When combined nitrogen is limiting,  $NH_4^+$  is incorporated through the glutamine synthetase–glutamate synthase pathway, while in the presence of combined nitrogen glutamic dehydrogenase is more active (Okon et al., 1976b).

It has been found that *A. brasilense* and *A. lipoferum* harbour several plasmids with a wide range of molecular weights (Franche & Elmerich, 1981; Wood & Dugan, 1981). However, no relationship between plasmids and phenotypic properties of *Azospirillum* has yet been established.

Although the extrapolation of activities and properties of *Azospirillum* in pure cultures to what is actually happening in the rhizosphere is highly speculative, the properties of *Azospirillum* reviewed above indicate the potential adaptability and survival capability of this organism in the constantly changing rhizosphere environment. Such organisms are likely candidates for use as inocula that may exert a significant influence on plant growth.

#### *Mode of Association with Roots and Experiments in the Greenhouse*

The *Azospirillum*–grass interaction does not produce visible structures on roots (Neyra & Döbereiner, 1977). Direct and indirect evidence showing azospirilla in close association with roots of grasses (associative symbiosis, diazotrophic rhizocoenoses) has accumulated (Döbereiner & De-Polli, 1980). By estimating numbers of *Azospirillum* (most probable number method) on washed roots before and after surface sterilization and crushing of the roots, significant populations ( $1 \times 10^5$ – $1 \times 10^8$

cells/g root), comprising about 1–10% of the total rhizosphere population, could be obtained from inoculated plants (Okon et al., 1977b; Okon et al., in prepn). Moreover, bacterial numbers do not express the full extent of *Azospirillum* contribution to root microbial biomass, since groups of cells or microcolonies are counted by serial dilutions as one bacterium, and *Azospirillum* cells are larger than cells of other gram-negative rhizosphere organisms (Döbereiner & De-Polli, 1980).

Light, fluorescent antibody and electron microscopy show azospirilla mainly embedded in the mucigel layer, but they are also present between cells and filling apparently dead cells in the cortex, endodermis, xylem and stele (Döbereiner & Day, 1976; Patriquin & Döbereiner, 1978). However, no correlation between *Azospirillum* populations on or inside the roots and nitrogen fixation activity has been clearly established.

In inoculated *Setaria italica*, highest numbers of *Azospirillum* associated with the roots were obtained during the booting stage (Okon et al., unpubl.). At this stage, highest acetylene reduction activities were obtained in intact inoculated plants (Kapulnik et al., 1981c; Okon et al., unpubl.).

By using the Fahraeus technique, it has been observed that millet seedling root hairs adsorb azospirilla in significantly higher numbers than cells of other  $N_2$  fixers (*Rhizobium*, *Azotobacter*, *Klebsiella*) (Umali-Garcia et al., 1980). Some degree of specificity between *A. brasilense* and roots of  $C_3$ -grasses (wheat) and *A. lipoferum* and roots of  $C_4$ -grasses (maize) has been proposed (Baldani & Döbereiner, 1980). However, no correlation of these findings with microbial activity or benefit to the plants investigated was demonstrated in the specificity system.

In pearl millet seedlings (Fahraeus technique), *Azospirillum* produces plant growth hormones (auxin, gibberellic acid, cytokinin) that increase the absorbing surface of the roots by producing more root hairs, lateral roots and mucilaginous sheath (mucigel) as compared to non-inoculated plants (Tien et al., 1979; Umali-Garcia et al., 1980). These observations have not been followed during plant ontogeny and the effect of hormones on yield has not been tested in the soil.

It has recently been found (Okon et al., unpubl.) that inoculation of *Zea mays* with *A. brasilense* strains Cd or sp. 7 significantly enhanced (30–50% over controls) the uptake of  $NO_3^-$ ,  $K^+$  and  $H_2PO_4^-$  into root segments from 2 and 3–4-wk-old plants. No apparent gross changes in root morphology could be observed in inoculated plants. However, root cross sections revealed changes in cell arrangement in the outer 4–5 layers of the cortex, probably increasing the surface actively involved in ion uptake. Moreover, inoculated corn plants had increased shoot dry weight by 20–30% over controls after three weeks. Similar increases in shoot dry weight were obtained in corn and sorghum plants grown to maturity in the greenhouse. In the above systems nitrogen fixation was suppressed by providing  $NO_3^-$ .

Tien et al. (1980) reported on the production of polygalacturonic acid transeliminase by *Azospirillum* species in cultures. Umali-Garcia et al. (1980) suggested that pectinolytic enzymes play an important role in root invasion by *Azospirillum*, which has been observed in intercellular parts of the root tissue. As a working hypothesis I propose that part of the *Azospirillum* benefit to plant development may be due to the

"softening" of the middle lamellae in the cortex, thus enhancing the mineral absorption surface of the cortex cells in a kind of "sponge" effect. It is assumed that no cell collapse occurs, as it does with pathogenic bacteria possessing strong pectinase activity.

Inoculation experiments with *Setaria*, maize and other grasses clearly demonstrated increases in plant dry weight (Albrecht et al., 1981) and total nitrogen content, above the amount initially present in isolated plant-soil systems (Nur et al., 1980; Cohen et al., 1981; Kapulnik et al., 1981b,c). High immediate acetylene reduction rates in *Setaria italica* were obtained mainly at reproductive stages in intact plant-soil systems incubated under high temperatures (30–32°C) (Cohen et al., 1980; Nur et al., 1980; Kapulnik et al., 1981c). Similar results were obtained in detached roots (Neyra & Döbereiner, 1977). The above observations have been further confirmed (Okon et al., in prepn) by following the patterns of growth and nitrogen fixation during the life cycle of *Setaria italica* (foxtail millet) inoculated with *Azospirillum brasilense*. Experiments were carried out under controlled environmental conditions in a growth chamber. The plants were fertilized at seeding time with a measured limiting amount of combined nitrogen and maintained on N-free mineral solution.

The inoculated plants gained significant amounts of nitrogen (15–25 mg N/plant) above that present in soil. During maturation of the plants, substantial nitrogenase activity of the intact root system was measured by the acetylene reduction method (up to 3500 nmole ethylene/h/plant). Concomitantly, nitrate reductase specific activities in the leaves decreased sharply at flowering indicating depletion of combined nitrogen in the soil, and high bacterial *Azospirillum* counts ( $1 \times 10^8$  per g dry wt of roots) could be measured. These systems were exposed to an  $^{15}\text{N}_2$  enriched atmosphere and increased levels of  $^{15}\text{N}$  in the roots and shoots indicated transfer of bacterially-fixed nitrogen to the plants.

Experiments with  $^{15}\text{N}_2$  (De-Polli et al., 1977) or by using the  $^{15}\text{N}$  isotope-dilution method (Rennie, 1980) also indicated benefit to the plant from the nitrogen fixed by *Azospirillum*. However, the way in which some of these experiments were carried out has been criticized (van Berkum & Bohlool, 1980). More convincing evidence has been presented by Eskew et al. (1981) in a rice-flooded soil system. By using  $^{15}\text{N}_2$  it was demonstrated that newly heterotrophically fixed nitrogen in association with the rice roots was available to the plant. However, other laboratories in the USA, such as in Oregon (Barber et al., 1976) and Maryland (Sloger & Owens, 1978), did not observe any marked benefit to the plants caused by inoculation with *Azospirillum*, due probably to different soil and environmental conditions used for testing the grass-bacterium association. One of the main problems encountered when working with *Azospirillum* in growth chambers has been cross-contamination of the controls (Albrecht et al., 1977).

Although it is now clear that grasses associated with *Azospirillum* (intact systems in the greenhouse) support vigorous acetylene reduction and nitrogen gains, more experiments using  $^{15}\text{N}_2$  are still needed to prove unequivocally that the plants benefit from the nitrogen that has been fixed.

#### *Inoculation with Azospirillum in the Field*

Early field experiments (Smith et al., 1976) showed that *Azospirillum* caused

yield increases (plant dry weight) in *Panicum* and *Pennisetum* mainly in soil fertilized with intermediate levels of nitrogen. However, no total nitrogen yield increases were demonstrated. Also, clear benefits to crop yield have been reported in Egypt and Belgium (Hegazi et al., 1981; Vlassak & Reynder, 1981), but not in Wisconsin (Albrecht et al., 1981). It is interesting to note that detached roots, obtained from several *Azospirillum*-inoculated fields both in Wisconsin (Albrecht et al., 1981) and Israel (Kapulnik et al., 1981a) always showed significantly higher acetylene reduction rates than roots obtained from non-inoculated fields. However, activities measured by the detached root assay could not be extrapolated to the actual activity taking place in the field.

Thirty inoculation experiments using *Azospirillum*-peat inoculants were carried out in Israel during 1978–1981 in commercial fields under diverse soil and environmental conditions, at different levels of combined nitrogen, in irrigated and non-irrigated fields. Detailed reports on the field experiments have been published elsewhere (Kapulnik et al., 1981a, 1982; Okon, 1981). Inoculation with *Azospirillum* was clearly beneficial to growth and commercial yield (increase of 10–35%) of several grain and forage grass crops in Israel (4 cultivars of corn, 2 of sorghum, 4 of wheat, *Setaria italica* and *Panicum miliaceum*). The benefit was observed in morphological and reproductive measurements of the different plants tested, and significant increases in the total nitrogen yield were obtained (Kapulnik et al., 1981a, 1982; Okon, 1981). Significant differences in yield (over controls) were obtained mainly in inoculated plots initially fertilized with intermediate levels of N. The yields were comparable to or higher than those obtained in the fully fertilized non-inoculated plots.

The results of the field experiments show that it is possible to exchange valuable nitrogen fertilizer for *Azospirillum* inoculation. Whether the mechanism by which the plants benefited in the field from *Azospirillum* inoculation is related to biological  $N_2$  fixation, to enhancement of available mineral uptake by roots associated with *Azospirillum* or to a combination of both processes is not yet known. It is possible that during the vegetative stage *Azospirillum* enhances root development and uptake of available minerals (N, P, K). Derepression of nitrogenase due to the gradually decreasing levels of combined nitrogen in the rhizosphere caused by leaching, denitrification and plant uptake during the growth season could increase  $N_2$  fixation by the associated bacteria during the reproductive stage and benefit the plant by supplying nitrogen (even a small amount) when it becomes a limiting factor. A similar pattern of balanced nitrogen supply during the growth season is known to benefit nodulated legumes (Hardy & Havelka, 1975).

The above working hypotheses based on results obtained in the greenhouse remain to be demonstrated under field conditions. Results from field experiments carried out in Israel clearly indicate much higher benefits to plants from inoculation with *Azospirillum* than has so far been estimated (van Berkum & Bohlool, 1980). It is possible that the high  $CaCO_3$  content and pH (7.5–8.4) of Israeli soils, together with prevailing high soil temperatures and light intensities, may favour the *Azospirillum* activities in association with grass roots in the field.

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