Potential Use of *Azospirillum* as Biofertilizer[1]

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ABSTRACT

Azospirillum was initially tested as a potential biofertilizer for cereals more than 15 years ago. Despite the optimistic initial results, *Azospirillum* inoculation in the field has proven to be inconsistent and unpredictable. Results were difficult to repeat even when experiments were performed identically, thus field experimentation with *Azospirillum* has been drastically reduced. It is presumed that *Azospirillum* inoculation of cereals should result in an average increased yield of 10-15% in fertilized areas and up to 20% under less developed agricultural practices. However, this is difficult to predict as long as basic features of the plant-bacteria interaction are unknown. Current research is focusing on two new directions in *Azospirillum* inoculation: (1) dual inoculation of *Azospirillum* and other rhizosphere microorganisms such as *Rhizobium*, pseudomonades and mycorrhizal fungi; the role of *Azospirillum* in this multiple interaction is that of a "helper" bacteria which improves the interaction of these microorganisms with plants; and (2) inoculation of non-cereal crop plants and ornamental plants. Since this unspecific bacteria effects a large variety of plants, it is possible that inoculation of non-cereal plants will produce more consistent results.

Key words: Plant growth-promoting rhizobacteria; plant-bacteria interaction.

RESUMEN

Hace más de quince años se probó, por primera vez, el potencial de *Azospirillum* como biofertilizante en cereales. A pesar del optimismo de los resultados iniciales, la inoculación con *Azospirillum* en el campo ha demostrado ser inconsistente e impredecible. Ha sido difícil reproducir los resultados, a pesar de que los experimentos se han realizado de manera idéntica. Consecuentemente, la experimentación de campo con *Azospirillum* se redujo dramáticamente. Estimaciones actuales proponen que la inoculación de cereales con *Azospirillum* debería incrementar el rendimiento de un 10%-15%, en áreas fertilizadas, hasta un 20% con el uso de prácticas agrícolas menos desarrolladas. Sin embargo, es difícil de predecir si se desconocen los factores básicos que intervienen en la interacción planta-bacteria. Las investigaciones se enfocan hacia dos nuevas direcciones: 1)Inoculación doble de *Azospirillum* con otros microorganismos de la rizósfera como *Rhizobium*, pseudomónades y hongos micorrizas. La función de *Azospirillum* en esa interacción múltiple es la de bacteria "cooperadora", pues contribuye positivamente en la interacción de estos microorganismos con las plantas. 2) Inoculación de plantas no-cereales y plantas de ornato. Esta bacteria no-específica produce efectos positivos en una gran variedad de plantas, por eso es factible que la inoculación en plantas no-cereales dé resultados consistentes.

Palabras clave: Rhizobacterias promotoras, crecimiento en plantas, interacción.

INTRODUCTION

After 50 years of obscurity, *Azospirillum* was rediscovered in the mid 1970s by J. Döbereiner and her colleagues in Brazil. At the time, it was considered by many to be the equivalent of *Rhizobium*, but to more economically important cereal plants. Consequently, this field of research was heavily financed by the biotechnology industry. However, within a few years, this promising "gold mine" had frustrated most investors. Even though field inoculation could increase the yield of many cereals up to 30% (and even more under greenhouse conditions), it failed to produce the con-

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sistent results required by the farming industry of developed countries. There emerged no "formula for success" that could be adopted for higher yields, and results were erratic and random.

Consequently, most research funds were withdrawn in the 1980s and redirected to the bio-control of soil-borne pathogens (Bashan and Levanony 1990; Jagnow 1987; Michiels *et al.* 1989). It has been clear from the beginning that in order to make a breakthrough in inoculation technology, one of the most important questions is how *Azospirillum* affects plant growth. Unfortunately, unlike rhizobia-producing nodules, *Azospirillum*-plant interaction produces no clearly visible phenotype in the root system. Therefore, the search for a mechanism is complicated and has been heavily influenced by personal interpretation. Over the last 15 years, several mechanisms of plant-microbe interaction have been proposed.

Nitrogen fixation

The first mechanism to be proposed was N_2 fixation, since this bacteria is an efficient nitrogen fixer (Pedrosa 1988). Much of the literature from 10-15 years ago demonstrated that *Azospirillum* inoculation significantly increased the total nitrogen of the plant. However, careful analysis of the bacteria's contribution to the fixed nitrogen showed that it was responsible for, at most, 18% of the accumulated nitrogen (Rennie and Thomas 1987). Other studies showed that the real nitrogen contributed by the bacteria was within 5% of the accumulated nitrogen (Okon *et al.* 1983). Furthermore, deletion of the genes for nitrogenase from the bacteria did not arrest the growth rate of inoculated tomato plants (Bashan *et al.* 1989c). Therefore, it is unlikely that N_2 fixation plays an important role for the plant, although it might play a role in the rhizocompetence of the bacteria.

Hormonal effects

A longstanding claim has been that the *Azospirillum* mechanism is based on changes in plant hormones induced by the bacteria. This theory is based on several facts: (1) this bacteria produces several plant hormones in culture, especially IAA, a fact known since the early years of research on this bacteria (Hartmann *et al.* 1983; Kucey 1988); (2) the application of synthetic hormones to plants produces effects that mimic the effect of *Azospirillum* inoculation; (3) hormone-overproducing mutants cause more pronounced effects on plant growth than wild-type strains; (4) changes in plant hormones have been detected, but in only one plant species so far (Fallik *et al.* 1989).

These facts provide indirect evidence that *Azospirillum* is involved in plant hormone regulation. However, this evidence alone cannot confirm hormonal effects as the principal mechanism by which *Azospirillum* promotes plant growth. Many unanswered questions remain: (1) Do changes in root morphology, presumably induced by hormones, have a direct effect on the growth of a plant and ultimately produce a higher yield? Hormonal changes that have only been observed at the seedling stage may not affect mature plants during the productive period several months later, when the *Azospirillum* population on the roots sharply declines. (2) Are irreversible IAA-deficient mutants, isogenic to the parental strain, incapable of producing morphological effects on roots? (3) Do various soil-grown plant species have similar changes in the hormonal balance?

General improvement in root growth and mineral uptake

In addition to its effect on roots, *Azospirillum* inoculation improved many plant foliage parameters which were attributed to improved mineral and water uptake (Murty and Ladha 1988). Evidence gathered from inoculated plants includes: enhanced accumulation of many minerals in plant foliage, enzymatic activities related to ion transformation in plant foliage, improved water uptake, partial substitution of nitrogen fertilization, and increased proton efflux.

Despite these visible effects, some crucial questions remain. It is likely that improved mineral and water uptake play an essential role in *Azospirillum*-plant interaction; however, it has not been shown whether this is the cause, or the result, of another mechanism such as a change in the hormone balance of the plant. Furthermore, the wide range of enzymes related to ion transport within the plants has been poorly studied, and no detailed analysis has been made of *Azospirillum* mutants that do not improve the mineral and water uptake in plants. Crucial to the full acceptance of this theory is that very few strains have been evaluated. It is doubtful that most *Azospirillum* strains possess these abilities, as some *A. brasilense* strains failed to improve the uptake of several ions, yet still improved plant growth (Bashan *et al.* 1990).

Signal molecules

A novel perspective has recently attempted to end the dead-lock on the mechanism of *Azospirillum*. The fact that *Azospirillum* affects plant cell metabolism from outside the plant (Bashan *et al.* 1991; Levanony *et al.* 1989) suggests that the bacteria are capable of excreting and transmitting signals which cross the plant cell wall and are recognized by the plant membranes. This interaction initiates a chain of events which result in the observed altered metabolism of inoculated plants. Since plant membranes are extremely sensitive to any change, they may serve as precise indicators of *Azospirillum* activity at the cellular level.

Signal molecules which enhanced proton efflux from roots and changed the membrane potential were detected in wheat, cowpea and soybean plants (Bashan 1990, 1991; Bashan *et al.* 1989a; Bashan *et al.* 1991). Although this is a promising avenue for future research, much remains to be clarified. Is there a relationship between the membrane activities of inoculated plants and growth parameters? Do these phenomena, detected *in vitro*, also occur *in situ*? What is the chemical nature of these molecules? Can different *Azospirillum* strains and plant species form an interaction which results in changes in membrane activity?

Additive hypothesis

Although the above proposals are based on experimental evidence, there are insufficient quantitative data to support the notion that one of these mechanisms is solely responsible for changes in plant growth. Therefore, we are submitting an "additive hypothesis": *Probably more than one mechanism participates in the association, either simultaneously or in succession. The sum of their activities, when introduced under the proper environmental conditions, results in the observed changes in plant growth.* This hypothesis may also explain the previously inconsistent results. Presumably, one or more mechanisms are inactive or only partially active, thus maximal benefits are rarely achieved. This hypothesis, may ultimately lead us to re-define *Azospirillum* as a "plant growth-promoting rhizobacteria" (PGPR) instead of an "associative nitrogen fixer."

Importance of attachment of *Azospirillum* to roots

The frequent failure of the inoculation experiments returned the focus of research to the most fundamental feature of this interaction: The bacteria does not produce any structural formation on the roots and therefore it is unprotected from environment and microbial competitors. To survive on the root surface, the bacteria must produce some permanent anchoring mechanism.

The secure attachment of beneficial bacteria is essential for a long-term association with the host plant for three reasons: (1) If the bacteria is not attached to root epidermal cells, substances extracted by the bacteria diffuse into the rhizosphere where they are consumed by nutritionally-versatile microorganisms before reaching the target plant. However, when the bacteria attach to the roots, part of these substances are diffused from their longitudinal side into the intercellular spaces of the root cortex. This is especially true for bacterial aggregate colonization where attachment is horizontal to the root surface (Levanony and Bashan 1991). (2) Without a secure attachment, water may wash the bacteria away from the rhizosphere to perish in the surrounding, nutrient-deficient soil. *Azospirillum* is known to survive poorly in many arid soils without plants to act as hosts (Bashan and Levanony 1990). (3) Association sites on roots with no attached beneficial bacteria are vulnerable to other aggressive, non-beneficial colonizers.

Current studies in several laboratories (Bashan and Holguin 1993; Bashan and Levanony 1988 a,b, 1989a; Bashan *et al.* 1986; Bashan *et al.* 1991; Del Gallo *et al.* 1989; Levanony and Bashan 1991; Michiels *et al.* 1991) have revealed the presence of fibrillar material of various dimensions connecting the bacteria to roots and sand surfaces. The chemical nature of this fibrillar material is still uncertain. Is it protein or polysaccharide (Bashan and Levanony

1988; Michiels *et al.* 1991), and how many different mechanisms exist? Findings in this basic research area will ultimately influence applicative studies in *Azospirillum* technology.

Mixed inoculation of *Azospirillum* with other microorganisms

Inoculation with *Azospirillum* alone has a limited future as long as the abovementioned questions remain. However, a new avenue of investigation has evolved that may breathe new life into *Azospirillum* technology: mixed inoculation. In this technology, *Azospirillum* is mixed with other microorganisms which have a proven effect on plants, such as *Rhizobium* and mycorrhizal fungi, in order to enhance the effectiveness of the latter (Barea *et al.* 1983; Del Gallo and Fabri 1991; Plazinski and Rolfe 1985). This role of "helper" bacteria is especially suitable for *Azospirillum* since its primary effect is on increased root development (higher surface area, more root hairs, and increased excretion of root exudates), thus increasing the probability of successful infection by the major contributor in a synergistic way.

The co-inoculation approach is currently the most promising one; however, data are still insufficient to justify full-scale field experiments with reasonable chances of success.

Inoculation of non-cereal crop plants

Azospirillum was initially isolated from cereals roots, and most inoculations have been done on cereals. However, the inconsistent results of *Azospirillum* inoculation on cereals invited researchers to evaluate the inoculation of other plant species. It appears that many other plant species react positively to inoculation, and they appear to do it more consistently (Bashan *et al.* 1989c; Puente and Bashan 1993). However, research in this area is still at the greenhouse stage, and field studies would be required to validate any claims of consistency.

Other difficulties inhibiting the commercialization of *Azospirillum* technology

The ultimate test for even the most beneficial isolate is its ability to survive and to successfully colonize plant roots in the presence of larger number of other indigenous rhizosphere microorganisms. The study of the bacterial behavior in various competitive environments (Bashan 1986c; Bashan 1991; Bashan and Levanony 1987, 1989b; Bashan *et al.* 1991; Harris *et al.* 1989) is in its infancy. No data are available on interaction between *Azospirillum* and the most prominent rhizosphere bacteria, let alone with fungi. Two major facts are known: (a) *Azospirillum* is not a biocontrol agent against soil-borne pathogens, as are many pseudomonades, and (b) suppression of competing microfauna encourages colonization by *A. brasilense* (Bashan 1986b).

Genetic research in *Azospirillum* is the Achilles heel of this system. A number of studies were conducted on *Azospirillum* genetics. Unfortunately, most of them were related to the nitrogen-fixing ability of the bacteria (Elmerich *et al.* 1989), which is meaningless to *Azospirillum* technology, as explained before. Recently, the first genes related to plant bacteria interaction were identified, and a few mutants were manipulated (Abdel-Salam and Klingmüller 1987; Vande Broek *et al.* 1989). Thus, it is evident that genetic manipulation to produce a super-*Azospirillum* is in the distant future and should not be considered as a feasible possibility for *Azospirillum* technology today.

Inoculant carriers for *Azospirillum* are no different from those produced for other beneficial bacteria used as biocontrol agents, or for *Rhizobium*. The first commercial *Azospirillum* inoculants were recently released (Fages 1991; Okon and Labandera-González 1994). Most *Azospirillum* inoculants are based on peat, vermiculite or various organic waste substances. These inoculants have many limitations, and to date the most advanced inoculant carriers are micro-capsules of *Azospirillum* in polymeric matrix. This inoculant carrier are currently under development in several laboratories (Bashan 1986a; Van Elsas and Heijnen 1990).

Conclusions

The facile exploitation of *Azospirillum* technology proved to be a costly dream which has discouraged research in this plant-bacteria interaction. However, during the last decade it has been repeatedly shown that, although complex, this system has the potential for agricultural exploitation. The main difficulty is our incomplete understanding of the basic system and several characteristics that are unique to this system. Alternatively, since the *Azospirillum* system has been one of the most studied in rhizosphere research, it is being used as a model for basic rhizosphere research regardless of its commercial potential.

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