Plant Growth-Promoting Effects of Rhizobacteria on Abiotic Stressed Plants. _Azospirillum_-Grasses Model

Cecilia M. Creus*, María A. Pereyra, Elda M. Casanovas, Rolando J. Sueldo, Carlos A. Barassi

Unidad Integrada Facultad de Ciencias Agrarias de la Universidad Nacional de Mar del Plata- EEA INTA Balcarce, CC 276, (7620) Balcarce, Argentina

Corresponding author: * creus@balcarce.inta.gov.ar

ABSTRACT

Plant growth-promoting rhizobacteria exert beneficial effects on plants when re-introduced by inoculation in a soil containing competitive microflora. Amongst them _Azospirillum_ is one of the most studied genera. Even though it colonizes different plant species in an ample variety of soils, it was first described in association with grass roots. In Argentina over 220,000 ha of wheat and corn were commercially inoculated with _Azospirillum_ in 2008. In this review the management conditions leading to enhanced crop productivity are discussed. The beneficial effects of inoculation on abiotic stressed plants are also described. We present results showing how wheat and maize stress tolerance are enhanced due to _Azospirillum brasilense_ Sp245 inoculation.

**Keywords:** inoculant, maize, plant-growth-promoting-rhizobacteria, water stress, wheat

**Abbreviations:** ACC, 1-aminocyclopropane-1-carboxylic acid; BNF, biological nitrogen fixation; IAA, indole-3-acetic acid; ISR, induced systemic resistance; IST, induced systemic tolerance; MPN, most probable number; NO, nitric oxide; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FA, fatty acid; PGPR, plant growth-promoting rhizobacteria; PEG, polyethylene glycol

**CONTENTS**

**IMPACT OF RHIZOBACTERIA ON AGRICULTURE** .......................................................... 49

**USE OF PGPR-BASED INOCULANTS IN ARGENTINA. EFFECTS AND CONDITIONS** .......................................................... 50

**RHIZOBACTERIA HELP PLANTS TOLERATE STRESS** ........................................................................................................ 51

**INOCULATION ON WHEAT AND MAIZE UNDER ABIOTIC STRESS: _AZOSPIRILLUM_-GRASSES MODEL** ..... 52

_Azospirillum_ the best studied PGPR .......................................................................................... 52

_Azospirillum brasilense_ Sp245 root colonization .................................................................. 53

Wheat and maize seedling growth promotion ...................................................................... 54

_Azospirillum_-grasses association in the field ....................................................................... 54

Mechanisms proposed to cope with water stress ................................................................ 54

**CONCLUSION AND PERSPECTIVES** ................................................................................. 56

**ACKNOWLEDGEMENTS** ...................................................................................................... 56

**REFERENCES** ........................................................................................................................ 56

**IMPACT OF RHIZOBACTERIA ON AGRICULTURE**

It is well recognized that the functionality of a terrestrial ecosystem depends on soil microbial activity (Doran 2002). Microorganisms in the rhizosphere react to the many metabolites released by plant roots. They also interact with plant roots by means of their own produced metabolites. In this scenario, signals derived from changes in the soil environment trigger selective root and shoot responses, as well as bacterial dynamic changes (Bais et al. 2006). Moreover, a sustainability analysis of crop production in terms of modern technological agriculture requires a detailed knowledge of the interrelationships between the microorganisms added to the system and those present in the soil. The result of these interactions would lead to a variety of positive, negative, and neutral effects on plants.

Among positive interactions, symbiotic ones between _Rhizobium_ spp. and leguminous plants are traditionally the most studied (Gray and Smith 2005). Nevertheless, associative interactions like those produced by free-living rhizobacteria and roots are acquiring greater interest. Since several decades ago, these bacteria have been the focus of research because non-leguminous plants like rice, wheat and maize are the most important crops feeding the ever-growing human population on our planet (Bashan and Hartmann 2009).

About 2 to 5% of rhizobacteria exert a beneficial effect on plant growth when re-introduced by plant inoculation in a soil containing competitive microflora, and are so called plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). They are free-living bacteria (Kloepper et al. 1989), and some of them invade the tissues of living plants and cause unapparent and asymptomatic infections (Sturz and Nowak 2000). PGPR may induce plant growth by direct or indirect modes of action (Beauchamp 1993; Glick 1995). Now, four mechanisms may be included into the direct mode. They are: 1) the production of phytohormones (Barbieri and Galli 1993; Dobbelaere et al. 1999) and stimulatory bacterial volatiles (Ryu et al. 2003); 2) the lowering of plant ethylene level (Glick et al. 1998); 3) the improvement of plant nutrient status by either making available those macro- and micronutrients from insoluble sources (Delvasto et al. 2006; Rodriguez et al. 2006; Hungria et al. 2010) or developing non-symbiotic nitrogen fixa-
tion (Boddey et al. 2008); and 4) the stimulation of disease-resistance mechanisms (induced systemic resistance, ISR) (van Loon 2007). Indirect effects arose when PGPR act like biocontrol agents leading to reduce diseases (Compañ et al. 2005), when they stimulate other beneficial symbioses, or when they protect the plant by degrading xenobiotics in inhibitory contaminated soils (Jacobsen 1997).

One important goal to improve agricultural performance and to increase crop production is to obtain high yields, even at low soil fertility or without intensive fertilization. To achieve this goal, the use of PGPR in cropping systems seems to be a reliable practice. From extensive genetic, biochemical, and applied studies, Azospirillum is considered one of the best-studied PGPR, and it has been regarded as a general plant root colonizer (Vande Broek et al. 2000; Bashan et al. 2004). Numerous studies all over the world have shown that the application of Azospirillum improves crop productivity. Azospirilla inoculants are able to exert beneficial effects on plant growth and yield of many agronomic crops under a variety of environmental and soil conditions (Bashan and Levanon 1990; Summer 1990; Fages 1994; Okon and Vanderleyden 1997). Even though there are many reports on successful experiments both in greenhouses and in the field, commercial application on large scale has lagged in the past decades (Bashan and Holguín 1994). Field and greenhouse experiments involving Azospirillum inoculation during the 1990’s were carried out in many countries including Israel, France, Belgium, Argentina, Uruguay, Mexico and South Africa. These experiments’ results were interesting reviewed by Okon and Labandera-Gonzalez (1994) and Dobbelaaere et al. (2001). They concluded that inoculation with Azospirillum resulted in significant yield increases in the magnitude of 5–30% in about 60–70% of the experiments. The beneficial effects were mainly observed in lighter soils under intermediate levels of fertilizer (N, P and K) and water regimes (Okon and Labandera-Gonzalez 1994). They also stated that successful inoculation experiments appear to be those in which researchers had paid special attention to the optimal number of cells in the inoculants, using appropriate inoculation methodology, whereby an optimal number of cells remained viable and available to colonize roots (Okon and Labandera-Gonzalez 1994; Dobbelaaere et al. 2001).

In the present decade, the use of commercial biological fertilizers is growing. Technically improved inoculants are now under development. They include formulations that can be used in place of chemical fertilizers is growing. Technically improved inoculants are now under development. They include formulations that can be used in place of chemical fertilizers (Dobbelaere et al. 2001). The chosen formulation determines potential success (Bashan 1998). The progress made in this aspect probably may permit to increase the frequency of positive results. Apart from this type of so-called first-generation inoculants, which include only native bacteria (wild type cells), studies on genetically modified microorganisms are being more and more common. These studies have demonstrated that it is possible to use genetic engineering to improve the properties of inoculants (Van Loon 2007). The interaction between inoculation and crop yield, water availability, and N and/or P-fertilization (biofertigation) showed that, as expected, fertilized wheat yield was enhanced with respect

USE OF PGPR-BASED INOCULANTS IN ARGENTINA. EFFECTS AND CONDITIONS

The leading countries in applying Azospirillum at the field are Mexico, with an estimated 300,000 inoculated ha in 2007 followed by Argentina, where over 220,000 ha of wheat and corn were commercially inoculated with Azospirillum in 2008 (Bashan and Hartmann 2009). The yield potential of agricultural soils and the quality of crops might be enhanced by incorporating some selected PGPR into the soils, enriching the indigenous microbial population, and/or optimizing some soil functional processes (e.g., nutrient cycling, energy flow) (Cabello-Mellado et al. 1992). In the last decades, there have been growing tendencies in agriculture with the development of PGPR’s methodologies and the fact that numerous bacterial-based inoculants developed for the main commercial crops are now available in the market (Maddoni et al. 2004), the fine tuning to achieve maximal efficiencies has not yet been attained (Díaz-Zorita et al. 2004). Pioneer studies in Entic Haplustols soils from the Pampas region of Argentina showed that wheat grain yield response to Azospirillum brasilense inoculation was dependent on differences in soil fertility and water availability (Rodríguez Cáceres et al. 1996). Even though several of these strains promoted greater tiller numbers, root dry matter and number of spikelets per plant, only INTA Az-8 or INTA Az-39 strains were able to promote a significant increase in grain yield (Puente et al. 2008). In this way, despite the lack of specificity in the interaction between the strain and the plant as in the case of a symbiotic relationship, a sort of potentiality amongst strains to develop different effects on crop production has been encountered. The INTA Az-39 strain was isolated from washed wheat roots originated in Marcos Juárez, Córdoba Province, Argentina (Rodríguez-Cáceres et al. 2008) and it is the native most studied strain. Inoculation with this strain resulted in a significant increase in wheat grain yield when evaluated in the semiarid region of Argentina (Rodríguez-Cáceres et al. 1996). In a large study conducted during 2002–2006 growing seasons, the performance of a commercial inoculant based on INTA Az-39 strain was evaluated in 297 experimental field trials in the Pampas region of Argentina (Díaz-Zorita and Fernández Canigia 2008). Crop technology were adopted at each on-farm location according to recommendations based on chemical soil analysis and suggested protocols for each local site. Wheat grain yield from those 297 experimental sites varied in a range from 850 to 8050 kg ha⁻¹ according to the management. The yield average increase was 260 kg ha⁻¹, equivalent to 8.0% of the mean wheat yield attained under the dry field farming conditions. The response of the experiments were determined in about 70% of the sites, depending mostly on the attainable yield and independently of fertilization and other crop and soil management practices. This is in agreement with the reported efficiency estimated from green house and field studies conducted in different parts of the world (Okon and Labandera Gonzalez 1994; Dobbelaaere et al. 2001). The interaction between inoculation and N and/or P-fertilization (biofertigation) showed that, as expected, fertilized wheat yield was enhanced with respect.

The Americas Journal of Plant Science and Biotechnology (Special Issue 1), 49-59 ©2010 Global Science Books
to that of the unfertilized crop. However, regardless of fertilization practice, inoculation significantly and positively affected yield, with mean yield responses of 259 and 260 kg ha⁻¹ for unfertilized and fertilized wheat, respectively. In general, the grain yield response to inoculation was greater at sites under sub-humid moisture regime, or with deep soil profiles, than under semi-arid environments or with shallow soils, respectively. Among the different soil types studied, only these soils subjected to both moisture reduction and shallows showed no significant grain production response to inoculation. This large assay allowed authors to conclude that in general, a greater response to inoculation is produced in the absence of major crop growth limitations, suggesting the complementary contribution of the A. brasilense treatment to more efficiently developing higher yielding wheat (Díaz-Zorita and Fernández Cangía 2008).

In a field trial conducted at the Estación Experimental Agropecuaria Balcarce of the Instituto Nacional de Tecnología Agropecuaria (INTA), we evaluated the response of A. brasilense Sp245-inoculated wheat to different water stress applications. The soil was an Argiudoll typic (5.4% organic content, 11.5 ppm P, 8.85 ppm NO₃-N, pH 5.7, with an average deeper of 80 cm). All plots were fertilized with (NH₄)₂HPO₄ at sowing based on soil analysis. Inoculation treatment significantly raised grain yield when water stress was gradually imposed during a period ranging from 15 days prior to 15 days after the phenological stage of anthesis. On the contrary, when the stress was applied from the onset of tillering, up to 15 days prior to anthesis, there were no inoculation positive effects on grain yield (Cresu 1997).

García de Salamone et al. (1992) carried out a field experiment on three commercial maize genotypes with two Azospirillum strains isolated from Argentinean soils and strain Sp7 isolated from Brazilian soils. They found a significant statistical interaction between strain, maize genotype, and fertilization. Subsequent two field experiments on Argentinean and Brazilian maize genotypes were carried out. The inoculant consisted of a mixture of four A. brasilense strains isolated from surface-sterilized maize roots in Argentina and three A. lipoferum strains isolated from surface-sterilized maize or sorghum roots in Brazil. Significant inoculation effects on total N accumulation and on grain yields were consistently negative with one Argentinean genotype and positive with four Argentinean and two Brazilian genotypes, indicating an unknown interaction between the plant genotype and bacterial strain. Positive results were detected on enhanced grain yield, harvest index and N harvest index (García de Salamone and Döbereiner 1996). Biological nitrogen fixation (BNF) contribution to N harvest increased in inoculated maize genotypes compared to non-inoculated maize genotypes. Inoculated maize grain yield and absorbed N varied between 7-43 and 5-46%, respectively, over non-inoculated controls values. Authors stated that their results strongly indicated that plant genotype-Azospirillum spp. strain interaction determines inoculation results on cereal crops. Inoculation’s N contribution to the analyzed soils was in the order of 100 kg N ha⁻¹. Capellelli et al. (2004) carried out a field experiment on two commercial Azospirillum strains isolated from Argentinean soils with N and P fertilization. Grain yield, biomass production, and roots length and area were improved by inoculation between 6-17, 10-22, and 12-18%, respectively.

In addition, rain-fed rice response to inoculation with two locally isolated strains of A. brasilense, namely S1 and S2, was evaluated in a field trial conducted in Tucumán Province, Argentina (Pedraza et al. 2008). The authors found that inoculation with both strains separately or in conjunction produced higher yield compared to non-inoculated controls, non-fertilized controls or inoculation combining both strains. Moreover, strain S2 produced the higher yield when it was applied together with urea fertilization. They also showed that even though urea-fertilization alone did not improve the total grain N content compared to the control, a significant improvement when applied together with different strains of Azospirillum was observed (Pedraza et al. 2008). Even though is well known that Azospirillum inoculated plants show a better nutrition (Bashan et al. 2004), there are yet scarce data that quantify the potentiality of this bacterium in Argentinean’s agricultural systems.

Altogether, these results point to a sort of threshold that limits the inoculants’ effects. Under this threshold, which can be generalized as a severe stress condition, crop yield shows no increase. When the environmental and management conditions overcome that threshold and the availability of resources begins to improve, the boost generated by inoculation on the yield increases in a range that depends on the management condition. Fig. 1 is a schematic representation of those conditions leading to different sized responses in yield.

**RHIZOBACTERIA HELP PLANTS TOLERATE STRESS**

In the biosphere, plants are often exposed to perturbations caused by the ambient where they live. It has been recognized that even though crop damage originated by pathogen attacks are the cause of substantial economic loses, the lack of water is the major threat against plant growth and crop production, even in humid areas (Boyer 1982). Coupled to the necessity for soil irrigation is the growing problem of field salinization worldwide. It was estimated that 10% of the world’s cropland and as much as 27% of the irrigated land might be already affected by salinity (Shannon 1997). The gradual increase in salt content in irrigated soils is a growing menace for crop production (Kotb et al. 2000). By the other hand, abiotic factors can also directly influence PGPR activity and probably their effect on plant growth and the dynamics of root microbial communities.

Most studies on PGPR interactions with other soil microorganisms and with soil fauna have been focused on biocontrol or ISR against fungal, bacterial and viral diseases and against insect and nematode pests. The role of PGPR as elicitors of ISR in plants is well established (Kloepper et al. 2004; van Loon et al. 2004). Some rhizobacteria can reduce the susceptibility to diseases caused by plant pathogenic fungi, bacteria, viruses and nematodes. Although ISR-eliciting rhizobacteria can induce typical early defense-related responses in cell suspensions, in plants they do not necessarily activate defense-related gene expression. Instead, they appear to act through priming of effective resistance mechanisms, as reflected by earlier and stronger defense reactions once infection occurs (van Loon 2007). An interesting review on the ISR by PGPR in crop plants underlines the potential of *Pseudomonas* species for commercial exploitation and the possibility to develop mixed inoculants against various pathogens attacking the same crop at a time (Ramamoorthy et al. 2001).

In addition to the possible ISR mechanisms, a healthy plant would obviously be more able to cope with pathogens...
than a weakened one. Not to mention that a general response of plants to *Azospirillum* inoculation is a better water status than in non-inoculated ones, mainly expressed under water and/or saline stress conditions (Creus et al. 1997; Casanovas et al. 2002, 2003; Creus et al. 2004; Barassi et al. 2007). However, the effects that PGPR mediate on physiological and biochemical changes in plants under abiotic stress are less reported. Recently Yang et al. (2009) proposed the term ‘induced systemic tolerance’ (IST) for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stress.

As a primary target, the root is the organ that shows the first stimulating bacterial effects. Indeed, the general effect upon inoculation with some PGPR is an increase in the development of the root system. One of the first observations regarding plant growth-promoting activity exerted by *Azospirillum* was on root size and morphology (Okon 1985). Based on these data, the possibility that a greater root system induced by rhizobacteria could lead to a greater exploration of soil resources, including water, was an interesting hypothesis established in the mid-1980’s (Okon 1985). Further studies demonstrated that the positive effects of *Azospirillum* were mainly derived from morpho-physiological changes of the inoculated plant roots with an enhanced capacity for water and mineral uptake (Okon and Kapulnik 1986). In fact, many subsequent studies demonstrated that *Azospirillum* inoculation improved plant growth under stress conditions (Okon and Labandera-González 1994; Creus et al. 1998; Casanovas et al. 2002, 2003; Creus et al. 2004; Bacilo et al. 2004; Kokalis-Burell et al. 2006; Nabi et al. 2010). After inoculation, the root displayed a significant increase in the number and length of root hairs, the rate of appearance and number of lateral roots, the diameter and length of lateral and adventitious roots, and the general root surface area (Kapulnik et al. 1985; Fallik et al. 1994; Dobelaere et al. 1999; Creus et al. 2005). Besides, Levanony et al. (1989) reported an increase in cell division in the root tips of inoculated wheat. In addition, some reports showed that the inoculation of wheat or maize seedlings with *Azospirillum* cells resulted in an increased number of root hairs showing a Y-shaped deformation (Patrikin et al. 1983; Jain and Patriquin 1984; Kapulnik et al. 1985; Zamudio and Bastarrachea 1994).

Also wheat, maize and sorghum showed higher absorption of N, P and K depending on the *Azospirillum* strain inoculated (Kapulnik et al. 1985; Morgersten and Okon 1987; Sarig et al. 1988; Hungria et al. 2010). Subjecting inoculated Sorghum bicolor plants to an osmotic stress in hydroponic systems diminished leaf senescence (Sarig et al. 1997).

On the other hand, the role of ethylene in the hormonal regulation of plant development has been well established. In spite of its effects on the development of some physiological processes like germination, a high level of ethylene concentration inhibits subsequent root elongation (Glick et al. 2007). During most phases of plant growth, ethylene production is minimal, but high levels may be synthesized as a defense mechanism against bacterial and fungal pathogens (Abel and Lee 1992). Under these circumstances, ethylene production increases (Abeles et al. 1992). Plants exposed to heavy metals and other stressors induce production of stress ethylene, which leads to premature plant senescence. In contrast, lowering ethylene levels mitigates harmful effects of many stressors (Reed et al. 2005). In addition, this molecule has been implicated in biotic stress, both as a virulence factor of fungal and bacterial pathogens and as a signaling compound in biotic stress (Abel and Lee 1992). One of the precursors of ethylene synthesis is 1-aminoacyclopropane-1-carboxylic acid (ACC). ACC deaminase is the key enzyme, commonly found in many soil microorganisms, capable of degrading ACC. Thus, lowering ethylene levels in plants can be considered as having potential for promoting growth (Glick et al. 1998). Indeed, PGPR containing ACC deaminase can help plants to resist certain stresses and grow in under hostile environmental conditions (Reed et al. 2005).

**INOCULATION ON WHEAT AND MAIZE UNDER ABIOTIC STRESS. AZOSPIRILLUM–GRASSES MODEL**

The *Azospirillum* genus is included in the alpha subclass of Proteobacteria belonging to the IV rRNA superfamily (Xia et al. 1994). This group of free-living microorganisms encompasses 13 species, each one classified according to its particular biochemical and molecular characteristics: *A. lipoforum* and *A. brasilense* (Tarrand et al. 1978); *A. amazonense* (Magalhães et al. 1983); *A. halopraeferens* (Reinhold et al. 1987); *A. irakense* (Khammas et al. 1989); *A. lipoferum* (Dekhil et al. 1997); *A. doebereinerae* (Eckert et al. 2001); *A. oryzae* (Xie and Yokota 2005); *A. melinus* (Peng et al. 2006); *A. canadensis* (Mehnaz et al. 2007a); *A. zeae* (Mehnaz et al. 2007b); *A. rugosum* (Young et al. 2008) and *A. pici* (Lin et al. 2009).

*Azospirillum* can fix atmospheric N, through the nitorgenase complex, when the availability of N compounds and oxygen tension are low (Döbereiner and Day 1976; Steenhoudt and Vanderleyden 2000). Even though this characteristic could be extremely valuable in agriculture, field studies including those in which isotopic dilution techniques were used, failed to demonstrate a significant BNF in *Azospirillum*-inoculated crops (Vande Broek et al. 2000). Even at the organism level, the growth promotion induced by the inoculation of axenic seedlings could not be ascribed to BNF (Bashan et al. 1989). Nevertheless, as was mentioned before, some experimental data demonstrated a significant BNF in maize crops (García de Salamone and Döbereiner 1996).

The production of phytohormones, namely auxins, cytokinins, and gibberellins, is the most commonly invoked mechanism of plant growth promotion exerted by PGPR. Among them, auxins are thought to play the major role. Even though it was suggested more than 60 years ago that auxin-like compounds could stimulate plant growth (Tien 1939), it was only in the 1970’s that this assumption was proved (Brown 1972; Barea and Brown 1974; Tien et al. 1979). Nowadays it is well known that *Azospirillum* can synthesize indole-3-acetic acid (IAA) by at least three different pathways. Controlled experiments in vitro showed that IAA content increased in roots and shoots of *A. brasilense* FT326-inoculated tomato (Ribaudo et al. 2006). To evaluate the involvement of bacterial IAA in the promotion of root development, several investigations were conducted with mutant strains altered in IAA production. Some experiments showed a reduced ability to promote root system development with different kinds of auxin mutants (Barbieri and Galli 1993; Kundu et al. 1997; Dobelaere et al. 1999).

However, there are no reports showing to what extent IAA is produced in the rhizosphere by *Azospirillum* (Lambrecht et al. 2008; Steenhoudt and Vanderleyden 2000). Moreover, the role of chemical signals in mediating rhizospheric inter-
actions is beginning to be understood (Bais et al. 2006). Among the signals that are potentially involved in root growth, N species as nitrate and NO (nitric oxide) are implicated in root growth and development (Lamattina et al. 2003). Creus et al. (2005) have reported the NO production by Azospirillum growing under aerobic conditions. The analogies found between the experimental data concerning Azospirillum stimulation of plant root development and the capability of NO to act as a non-traditional plant growth regulator (Beligni and Lamattina 2001) promoting adventitious root formation, lateral root development, and root hair formation, led us to explore whether Azospirillum ability to promote root growth and modify root architecture relies on NO. Recently we demonstrated that when NO is removed from the interaction between root and Azospirillum, both lateral and adventitious root formation were inhibited and attained to the non-inoculated values, evidencing that NO is strongly involved in the Azospirillum-induced root branching (Molina-Favero et al. 2007, 2008).

Other special characteristics present in Azospirillum make it a valuable PGPR. Some of them are briefly summarised below.

The bacterium is established mainly on the root surface but some strains of A. lipofirerum and A. brasilense are endophytes colonizing the apoplast and intercellular spaces of the root. This ability could mean a lower vulnerability to harsh conditions imposed by the environment (Sturz and Nowak 2000). Indeed, it has been published that A. brasilense and A. halopraerferans can withstand growth in seawater for more than 30 days (Puente et al. 1999). Azospirillum brasilense Sp245 (EMBRAPA-Rio de Janeiro, Brazil) initially isolated from sterilized wheat roots (Baldani et al. 1993) shows a high colonizing potential, and can be found in different parts of the root, including the inner root tissue, forming micro-colonies in intercellular spaces (Bashan et al. 2004). We evaluated the growth of A. brasilense Sp245 cells in N-free semi-solid media supplemented with either 160 mM NaCl, 320 mM NaCl, 20% polyethylene glycol (PEG) 6000, or 30% PEG 6000. Bacterial produced typical white growth pellicles under the surface showing in addition that the N-fixing capability is functional under these circumstances (Creus et al. 1997). Our findings also showed that A. brasilense Sp245 could tolerate up to 20% PEG 8000 in a chemically defined liquid medium (OAB broth) without appreciable decline in cells number (data not published). Azospirillum spp. can accumulate compatible solutes, mainly trehalose, proline, and glutamate depending on N source (Madkour et al. 1990) to allow adaptation to fluctuations in soil salinity or osmolarity (Bashan et al. 1997). This property is not conserved in the root-colonizing agent of soil-borne plant pathogens. However, there have been reports on moderate capabilities of A. brasilense in biocontrolling some different pathogenic agents (Bakanchikova et al. 1993; Sudhakar et al. 2000; Bashan and de-Bashan 2002). These antibacterial activities of Azospirillum could be related to its already known ability to produce bacteriocins (Oliveira and Drozdowicz 1987) and siderophores (Arsac et al. 1998; Shah et al. 1992) or phenylacetic acid, an auxin-like molecule with antimicrobial activity (Somers et al. 2005).

As the most researched associative bacterium, Azospirillum has become a cornerstone of rhizosphere research unrelated to its direct agricultural application. Azospirillum is an excellent model for studies of plant-associative bacteria in general. In nature, a broad host range may help bacteria to adapt to a greater variety of environmental conditions. Reports about Azospirillum from graminaceous plants were very common in the past, and so claims of Azospirillum specificity for certain cereal species were frequently published (Bashan and Levano 1990). They stated that Azospirillum mainly enhanced growth of cereal plants. The data published in recent years, however, show otherwise. They show that the bacterium is a natural inhabitant of many non-graminaceous plants. Azospirillum strains had no preference for crop plants or weeds, or for annual or perennial plants, and can be successfully applied to plants that have no previous history of Azospirillum in their roots. It appears that Azospirillum is a universal bacterium found almost everywhere, a general root colonizer and is not a plant specific bacterium (Bashan and Holguin 1997). The full host range of Azospirillum has not yet been defined. We propose the grasses-Azospirillum interaction model, in an attempt to generalize the plant promotion response under stress conditions, after twenty years of studies on the effects of Azospirillum brasilense Sp245 inoculation in wheat and corn.

Azospirillum brasilense Sp245 root colonization

A successful Azospirillum colonization either of the rhizosphere, the surface and/or the interior of the root is determinant to enhance plant growth and crop yield (Dobbelaere et al. 1999). Several authors have reported that the expression and the magnitude of the plant-bacterium interaction would depend on the number of cells that colonize the root, and this number differs according to the plant species. The bacterial establishment is affected by various edaphic, abiotic and biotic factors, which also have direct influence on the plant growth and the functioning of the root (Vande Broek and Vanderleyden 1995). The inoculation method must ensure the liberation of the inocula in the field in a timely and precise way, allowing the bacterial cells to remain viable and to keep their ability to colonize the roots in an adequate number (Okon and Labandera-González 1994). This number varies according to plant species, being the recommended one 10-10^5 bacteria per plant 2-3 weeks after sowing in wheat (Kapulnik et al. 1985) and 10^5 in the case of maize (Arsac et al. 1990).

In our lab, we developed a simple method to obtain inoculated seeds containing high number of viable Azospirillum cells avoiding the use of external carriers or adhesives (Creus et al. 1996). Wheat seeds inoculated during imbibition and dried to 14% water content retained 3.7 × 10^6 viable cells g^-1 dry weight up to 27 days after bacterial treatment (Creus et al. 1996).

High cell concentrations in the roots might cancel out the growth promoting effect (Dobbelaere et al. 1999). A significant decrease in the size of the elongation zone of the roots was reported at higher inoculum levels (10^6 bacterial cells ml^-1) (Dobbelaere et al. 1999). Our results showed that since A. brasilense Sp245 could have been multiplying inside the plant roots, root-colonization should be taken into account instead of inoculum concentration (Pereyra et al. 2007).

Root colonization, expressed as most probable number of diazotrophic bacteria (MPN) should not exceed 2.5 × 10^5 bacteria Sp245 cells in T. durum cv. ‘Buck Topacio’ seeds, since 3.3 × 10^6 cells were colonizing seedlings roots, no growth promoting effects were detected (Pereyra et al. 2007). We concluded that to diminish the risks of overloading the roots and loosing the beneficial growth promoting effects exerted by A. brasilense Sp245 it would be advisable to use an inoculum containing less than 5 × 10^6 bacterial cells per seedling in cv. ‘Buck Topacio’ of durum wheat (Pereyra et al. 2007).

Not only high but also low cell concentrations in the roots might cancel out the growth promoting effect (Dobbelaere et al. 1999). Mild osmotic stress (-0.54 MPa) altered A. brasilense Sp245 wheat seedlings interactions, diminishing total root colonization in one order (10^6 to 10^5 cells g DW roots^-1) (Pereyra et al. 2006). In spite of this, Azospirillum inoculation promoted significant increases in coleoptile length, elongation zone, and projected area, and in root length (Pereyra et al. 2006).

Maize seeds were inoculated with 10^7 A. brasilense Sp245 cells seed^-1, air-dried to 14% humidity and dry stored at 15-20°C in the dark up to 25 days. After 0, 5, 15 and 25 days of storage seed were germinated and seedlings grown for 5 days. MPN in roots of seedlings from inoculated seeds decayed from 10^5 cells g^-1 at day 0 to 10^3 cells g^-1 at day 25. However the growth promoting effect on root and coleoptile was maintained (Casalnove et al. 2000).
Another important aspect to achieve proper root coloni-
zation, in view of the high input of agrochemicals in con-
temporary crop production, is the compatibility between
\textit{Azospirillum} inoculants with common pesticides. Our re-
results in laboratory tests showed that \textit{A. brasilense} Sp245
cells growth was not hampered by Tebuconazole, a fungi-
cide widely used to prevent the most common fungal dis-
eases in wheat. No effects were detected during expo-
ence (34.1 h) with the best performed bacteria in Aus-
tropic optimum conditions to interact with and colonize
seedling roots (Pereyra \textit{et al.} 2009). Tebuconazole also did
not affect \textit{A. brasilense} Sp245 wheat root colonization when
present in the hydroponic media in either normal or water
stress conditions (Pereyra \textit{et al.} 2009). Opposite results
were found by Puente \textit{et al.} (2008) in Tebuconazole treated
\textit{A. brasilense} Az39 INTA inoculated wheat seeds which pre-
sented a lesser number of cells than non treated controls.

\textbf{Wheat and maize seedling growth promotion}

The lack of water is one of the main constraints to plant
growth and crop yield. Many reports indicate that the best
performance of \textit{Azospirillum} in the field is associated with
non-optimal conditions for plant growth (Bashan \textit{et al.}
2006), where a noticeable improvement in the plant
performance of \textit{Azospirillum} inoculants with common pesticides. Our re-
results in Argentina (Díaz-Zorita and Fernández Canigia
2008). It has shown a high survival rate in seeds up to a
year, and no side effects when the seeds were previously
controlled with Carbendazim+Thiram; Tebuconazole or Tritic-
onia (Díaz-Zorita \textit{et al.} 2004). The inoculums’ formula-
tion and application technology are crucial for the develop-
ment of commercial \textit{Azospirillum} inoculants. Up to now,
relatively few studies have addressed the application of
\textit{Azospirillum} technology under practical agricultural condi-
tions (Díaz-Zorita and Fernández Canigia 2008). Puente
\textit{et al.} (2005) found that \textit{A. brasilense} Az39 INTA inoculated
wheat showed an increased grain yield, with an increase in
the number of tillers and spikelets and in the roots dry
weight at harvest. As it was mentioned Rodriguez Cáceres
\textit{et al.} (1996) suggested that \textit{Azospirillum}-inoculated wheat
colonization in the field is associated with increased root
surface. In this view, nutritional improvement of maize plants due to
\textit{Azospirillum} inoculation, could account for the amelioration of the harmful effects of
drought during the flowering period in both grain yield and
harvest index (Casnovas \textit{et al.} 2003).

\textbf{Mechanisms proposed to cope with water stress}

Despite the numerous studies on physiology and molecular
biology of \textit{Azospirillum}, there is no definite agreement on
exactly how the bacteria affect plant growth, which mecha-
nisms are involved, or if there is one major mechanism res-
ponsible for the observed effects on plant growth, particu-
larly, on plant yield (Bashan \textit{et al.} 2004). These questions
are ways far the driving force in the \textit{Azospirillum} research
field.

The production of phytohormones has been proposed as the
key feature of this bacterium and the cause of the altered
metabolism and morphology of the plant, yielding better
mineral and water absorption (Dobelaere \textit{et al.} 1999). The
most apparent outcome after inoculation is the production of
morphological changes in the root system. Inoculated plants larger roots would absorb minerals and water better
(Okon 1985). The increased root development leads to an
increased root surface that could improve plant nutrition
and thus would be a key factor for plant growth promotion
by PGPR in general. In this sense, developmental changes
promoted in roots must be triggered prior to the changes in
uptake of nutrients. This widely accepted hypothesis also
states that nutrient uptake would be increased over time
together with increased root surface. In this view, nutritional
improvement by PGPR would be an indirect consequence

\textbf{Azospirillum-grasses association in the field}

We studied the connections between water relations and the
yield in \textit{A. brasilense} Sp245-inoculated wheat suffering
drought during anthesis (Creus \textit{et al.} 2004). A lesser loss
(16.9\%) of grain yield was obtained in \textit{Azospirillum}-inocu-
lated plants suffering drought. The relative increase observed
was mainly due to the bacterial effect on the number of
gains per square meter (Creus \textit{et al.} 2004). We also studied
the mineral content of these grains. They showed higher Mg,
Ca and K contents. Neither drought nor inoculation changed
grain P, Cu, Fe and Zn contents. In this sense, inoculation
would be increasing the cost-benefit ratio, as the \textit{Azospiril-
lum} effect could promote not only a higher grain yield but
also a higher seed quality. For example, a higher than nor-
mal concentration of Ca in seeds, as that promoted by \textit{Azos-
spirillum} inoculated in water stressed plants, could mean
future seedlings with better performance under drought. As
it was mentioned above, \textit{A. brasilense} Az 39 INTA initially
isolated from Argentine pampas, has been described as the
strain with the best performance in \textit{Azospirillum} inoculants
in Argentina (Díaz-Zorita and Fernández Canigia
2008). Roots from 5-days-old seed-inoculated seedlings
grown in hydroponics showed a 63\% increase in dry weight
in the presence of Tebuconazole, even when exposed to 20% 
PEG 8000 solutions (Pereyra \textit{et al.} 2009). Tebuconazole also did
not affect \textit{A. brasilense} Sp245 wheat root colonization when
present in the hydroponic media in either normal or water
stress conditions (Pereyra \textit{et al.} 2009). Opposite results
were found by Puente \textit{et al.} (2008) in Tebuconazole treated
\textit{A. brasilense} Az39 INTA inoculated wheat seeds which pre-
sented a lesser number of cells than non treated controls.

\textbf{Wheat and maize seedling growth promotion}

The lack of water is one of the main constraints to plant
growth and crop yield. Many reports indicate that the best
performance of \textit{Azospirillum} in the field is associated with
non-optimal conditions for plant growth (Bashan \textit{et al.}
2006), where a noticeable improvement in the plant
performance of \textit{Azospirillum} inoculants with common pesticides. Our re-
results in Argentina (Díaz-Zorita and Fernández Canigia
2008). It has shown a high survival rate in seeds up to a
year, and no side effects when the seeds were previously
controlled with Carbendazim+Thiram; Tebuconazole or Tritic-
onia (Díaz-Zorita \textit{et al.} 2004). The inoculums’ formula-
tion and application technology are crucial for the develop-
ment of commercial \textit{Azospirillum} inoculants. Up to now,
relatively few studies have addressed the application of
\textit{Azospirillum} technology under practical agricultural condi-
tions (Díaz-Zorita and Fernández Canigia 2008). Puente
\textit{et al.} (2005) found that \textit{A. brasilense} Az39 INTA inoculated
wheat showed an increased grain yield, with an increase in
the number of tillers and spikelets and in the roots dry
weight at harvest. As it was mentioned Rodriguez Cáceres
\textit{et al.} (1996) suggested that \textit{Azospirillum}-inoculated wheat
colonization in the field is associated with increased root
surface. In this view, nutritional improvement of maize plants due to \textit{Azospirillum} inoculation, could account for the amelioration of the harmful effects of
drought during the flowering period in both grain yield and
harvest index (Casnovas \textit{et al.} 2003).

\textbf{Mechanisms proposed to cope with water stress}

Despite the numerous studies on physiology and molecular
biology of \textit{Azospirillum}, there is no definite agreement on
exactly how the bacteria affect plant growth, which mecha-
nisms are involved, or if there is one major mechanism res-
ponsible for the observed effects on plant growth, particu-
larly, on plant yield (Bashan \textit{et al.} 2004). These questions
are ways far the driving force in the \textit{Azospirillum} research
field.

The production of phytohormones has been proposed as the
key feature of this bacterium and the cause of the altered
metabolism and morphology of the plant, yielding better
mineral and water absorption (Dobelaere \textit{et al.} 1999). The
most apparent outcome after inoculation is the production of
morphological changes in the root system. Inoculated plants larger roots would absorb minerals and water better
(Okon 1985). The increased root development leads to an
increased root surface that could improve plant nutrition
and thus would be a key factor for plant growth promotion
by PGPR in general. In this sense, developmental changes
promoted in roots must be triggered prior to the changes in
uptake of nutrients. This widely accepted hypothesis also
states that nutrient uptake would be increased over time
together with increased root surface. In this view, nutritional
improvement by PGPR would be an indirect consequence

\textbf{Azospirillum-grasses association in the field}

We studied the connections between water relations and the
yield in \textit{A. brasilense} Sp245-inoculated wheat suffering
drought during anthesis (Creus \textit{et al.} 2004). A lesser loss
(16.9\%) of grain yield was obtained in \textit{Azospirillum}-inocu-
lated plants suffering drought. The relative increase observed
was mainly due to the bacterial effect on the number of
gains per square meter (Creus \textit{et al.} 2004). We also studied
the mineral content of these grains. They showed higher Mg,
Ca and K contents. Neither drought nor inoculation changed
grain P, Cu, Fe and Zn contents. In this sense, inoculation

The America Journal of Plant Science and Biotechnology 4 (Special Issue 1), 49-59 ©2010 Global Science Books
of their effect on root development (Mantelin and Touraine 2004). Nevertheless, direct effects on root transport systems cannot be ruled out. Bertrand et al. (2000) showed that an Achromobacter sp., enhanced NO3 uptake rate per unit of root area in Brassica napus roots, and Saubidet et al. (2002) reported that the inoculation with A. brasilense increased the N content of wheat plants. Our results showed that inoculated wheat seedlings had larger roots than non inoculated controls either in well irrigated conditions or when exposed to 20% PEG 8000 solutions (Pereyra et al. 2006, 2009).

It was proposed bacterial direct effects on plant cell membranes as an alternative mechanism. Bashan et al. (1992) showed that soybean (Glycine max) and cowpea (Vigna unguiculata) inoculated with A. brasilense enhance H+ extrusion from their roots compared to the normal extrusion occurred in non-inoculated plants. After nine hours of transferring cowpea plants growing in hydroponic solutions to a new one the pH of the media decreased from 6 units of pH to the range of 4.13 ± 0.19 units, providing more evidence about a direct effect on root cell membranes. The bacteria affect several plant metabolic pathways, including cell membrane activity. To shed light on the possible relationship between the alleviation effects of water stress on wheat, and the lipid composition of roots, we studied Azospirillum-inoculated wheat seedlings root phospholipids (PL) composition, fatty acid (FA) distribution profiles, and degree of unsaturation of major PL classes, from seedlings growing in darkness under osmotic stress (Pereyra et al. 2006). When both Azospirillum inoculation and water stress treatments were combined, the distribution of the major PL classes (phosphatidylcholine (PC) and phosphatidylethanolamine (PE) remained unaltered, while FA distribution profiles changed in both PC and PE. We concluded that Azospirillum inoculation could contribute to protect wheat seedlings from water stress through changes in the FA distribution profiles of the major PC and PE root PL (Pereyra et al. 2006). Other unpublished experiments performed in our laboratory confirmed that among the FA affected by inoculation followed by stress, the linoleic acid (18:2) content of PC increased the most (Fig. 2). As was mentioned, many studies have suggested the involvement of auxins produced by Azospirillum on the root morphology (Bashan et al. 2004). These hormones are involved in the expression of ER-bound Δ12 desaturase, a key enzyme in the synthesis of 18:2. As a matter of speculation, this FA could be contributing to regulate the activity of key membrane-associated enzymes such as ATPases via modification of their lipid microenvironment (Palta 1990). Plasma membrane H+-ATPases pump protons from the cytoplasm to the apoplastic space, where extensive acidification is believed to contribute to cell wall loosening, a prerequisite for cell growth (Rober-Kleber et al. 2003).

Plants have developed mechanisms to respond to adverse environmental conditions that in some instances permit to repair the damage caused by stress. The maintenance of tissue turgor pressure is essential to several crucial processes upon which plant life depends. Higher plants have developed adaptive mechanisms to cope with water deficit. One of the most recognized mechanisms is osmotic adjustment, which involves active solute accumulation in plant tissues thus enabling plants to extract water at low soil water potential and maintaining cell turgor (Morgan 1984). Could it be possible that Azospirillum inoculation affect the stress tolerance mechanisms that trigger plants under stress? In the search for the answer to this question, we developed assays to measure the production of osmotics in root and leaf of inoculated maize. We demonstrated an improved water status and both foliar and radical high free proline concentrations in inoculated maize seedling with A. brasilense Sp245 (Casanovas et al. 2002). Seedlings also showed increased root growth, total aerial biomass and foliar area. Proline is usually considered as an osmoprotectant agent and their accumulation appears to render membranes more enduring (Sairam and Saxena 2000). However, some authors suggested that proline accumulation in vegetable tissues could be only useful as possible drought injury sensor instead of its role in stress tolerance mechanism (Vartaniam et al. 1992; Zlatev and Stoyanov 2005). In addition, proline is also known to be involved in reducing the oxidative damage by scavenging and/or reducing the free radicals, involved in tolerance mechanism against oxidative stress, which is in addition, the main strategy of plant to avoid detrimental effects of water stress (Vendruscolo et al. 2007).

On the other hand, in a hydroponic system, were no nutrients were present, A. brasilense Sp245 inoculation could also partially reverse the negative effects that water stress had on wheat seedlings, as it was observed in the growth rate of coleoptiles (Alvarez et al. 1996). Furthermore, as a correlation between coleoptile length and osmoregulation among wheat genotypes has been reported (Morgan 1988), we studied the impact of Azospirillum-inoculation on plant water relations (Creus et al. 1998). An overall view of these properties can be obtained in the same tissue by pressure-volume curves (Hellkvist et al. 1974). In this sense, the ability of a given plant cell to tolerate a restricted water supply depends on three known physiological mechanisms of adaptation: i) active or passive solute accumulation in vacuoles, ii) changes in cell wall elasticity, and iii) changes in the relative partitioning of water into apoplastic and symplastic fractions (Girma and Krieg 1992). We found differential effects of Azospirillum on the wheat seedlings’ capability to withstand water stress between different genotypes (Creus et al. 1998). Nevertheless, results were consistent with a better water status in Azospirillum inoculated wheat seedlings under water stress were both effects on cell wall elasticity and/or apoplastic water were evident (Creus et al. 1998). Additionally, the better water status (significantly higher water content, relative water content, water potential, apoplastic water fraction, and lower cell wall modulus of elasticity values) and the additional elastic adjustment found

![Fig. 2 Fatty acid composition (percentages) in phosphatidylcholine extracted from Azospirillum-inoculated wheat seedlings roots grown under normal or water stress conditions. Seedlings were grown in the dark and inoculated either with previously autoclaved A. brasilense Sp245 cells (control), or with 10^7 live bacteria per plant. Each of these groups was then grown for 2 days, either in sterile distilled water or in 20% PEG 8000 (stress).](image-url)
in *Azospirillum* inoculated wheat plants in the field (Creus *et al.* 2004) could be crucial in promoting higher grain yield and mineral quality at harvest, particularly when drought strikes during anthesis.

Biotic and abiotic factors that affect cell wall metabolism will influence its dynamic (i.e. expansion rate and cell growth). We reported that *A. brasilense* induced a more elastic cell wall and a higher apoplastic water fraction in white clover and flag leaf (Creus *et al.* 2004). These biophysical characteristics account for an improved response to stress and could permit increased growth even in water stressed plants. Knowledge on the biochemical effects the bacteria could elicit into plant cell wall and how these responses could change the hypocotyl physiology is still scarce. Our first results showed that the cell wall is a target for *Azospirillum* growth promotion (Creus *et al.* 2008; Pereyra *et al.* 2010).

Yet, Cassán *et al.* (2009) proposed another possible mechanism involved in coping water stress. They showed that *A. brasilense* Az239 was capable of producing cadaverine in chemically defined medium and that when present in rice seedling roots it promoted root growth and helped mitigate osmotic stress. They speculated that these effects were due in part to cadaverine production. Polyamines are considered as plant growth-regulating compounds; and among them, cadaverine has been correlated with root growth promotion or osmotic stress mitigation in some plant species. It was mentioned above that no unique and definitive mechanisms could be ascribed to the improved performance of grasses crops under stress. The best explain that was hypothesized since almost twenty years ago and is still alive is the Additive Hypothesis that considers multiple mechanisms in the intimate association between roots and bacteria which operate simultaneously or in succession (Bashan and Levanony 1990). The sum of their activities, when induced under appropriate environmental conditions, results in the observed changes in plant growth and crop yield.

**CONCLUSION AND PERSPECTIVES**

Great efforts all around the world are being made in many scientific programs in order to develop new crop varieties with enhanced drought and salt tolerance and better nutritional value through the introduction of desirable traits either by conventional breeding or genetic modification. However, the focus generally lay on the plant moiety, ignoring what is occurring with the other moiety of the interaction: the microbial communities of the soil. The important role of rhizobacteria that interact with plant roots and influence plant health, productivity and biodiversity must be included.

Traditional microbial population studies, based on identification, quantification, and the measurement of processes that occur in the rhizosphere, are often difficult or tedious. Similarly, the collection of relevant samples or the simulation of natural conditions in the laboratory can be problematical. However, with the array of molecular techniques that are becoming available, significant improvements in our understanding of the rhizosphere microbial communities and processes are anticipated (Barea *et al.* 2005; Johnson *et al.* 2008; Bakanchikova *et al.* 2010).

Primary conditions make survival difficult for the introduced bacteria. Harsh conditions, including frequent droughts, lack of sufficient irrigation, high salinity and soil erosion, may quickly diminish the population of any bacteria introduced into the soil unless precautions are taken to select the proper inoculant and provide irrigation concomitant with inoculation. However, in agricultural systems in developing countries, beneficial microorganisms may make the greatest contribution, if inexpensive and easy-to-use formulations can be developed (Bashan 1998). *Azospirillum*, as the most studied PGPR (Bashan *et al.* 2004), could provide valuable information that could help to expand its agrobiotechnological applications, as well as for a better understanding of other potentially useful microbial-plant associations.

The *Azospirillum*-based product application in agriculture is supported mainly by their effect as plant growth promoters and their improvement of nutrient assimilation. The adoption of this inoculation practice in association with recommended doses of fertilizers improve the crop tolerance to abiotic stresses without loss of productivity (Divan Baldani *et al.* 2008).

**ACKNOWLEDGEMENTS**

This work was supported by Secretaría de Ciencia y Técnica (SECyT), National University of Mar del Plata, Argentina.

**REFERENCES**


Barbieri P, Galli E (1993) Effect on root development of inoculation with an *Azospirillum brasilense* mutant with altered indole-3-acetic acid pro-ductions. Research in Microbiology 144, 609-625


Bashan Y, Levanony H (1990) Current status of *Azospirillum* inoculation tech-

©2010 Global Science Books
Azospirillum and grasses stress tolerance. Creus et al.
principales características. In: Centro Internacional de Mejoramiento de Maíz y Trigo (Eds) Trigo en el Tercer Mundo, México, pp 1-12.


Jacobsen CS (1997) Plant protection and rhizosphere colonization of barley by seed inoculated herbicide-degrading Burkholderia (Pseudomonas) cepaciae DB001 on rhizodegradation. Soil Biology and Biochemistry 29, 139-144.


