Azospirillum spp., a Dynamic Soil Bacterium Favourable to Vegetable Crop Production

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ABSTRACT

The soil is not merely an inert support for anchoring the plant. Its complexity and dynamics determine the way plants will grow and develop. In these processes, the root and its interaction with the different abiotic and biotic soil components represent a key point in the acquisition of water and essential nutrients. However, anthropogenic effects on the environment – including soil and water deterioration and contamination – could alter these relationships. In addition to these, vegetable production presents diverse problems, which could be mitigated by the use of plant-growth promoting microorganisms (PGPMs). On the soil, PGPMs could contribute to solubilize and/or to acquire essential minerals, making scarce nutrients more available to the plant. On the host, they stimulate several physiological changes that could lead to a better growth and to a plant more tolerant to abiotic stresses. Amongst PGPMs, *Azospirillum* is one of the most studied genera. Even though it colonizes different plant species in an ample variety of soils, its favourable effects on vegetable germination, emergence and growth, have not been thoroughly studied. This review describes the beneficial effects PGPM inoculation could have on vegetables growing either under normal or stressful conditions, with an emphasis on the use of *Azospirillum*. It also focuses on the recent advances on *Azospirillum*-plant interactions and the bacterial mechanisms of plant growth promotion.

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INTRODUCTION

Animals can move from one environment to another, choosing the most convenient according to their living requirements. Terrestrial plants in turn, can only make the best use of their place, adapting to the site where they live, to the environmental changes, and to the living organisms that interact with them. Indeed, terrestrial plants thrive in the aboveground-belowground interface. They are extraordinarily adapted to extract energy and nutrients from both environments. As energy and carbon are taken by the aerial part of the plant and water plus minerals come from the soil, plants rapidly differentiate in two main tissues: shoots and roots. At first sight, plants do not seem to have as complicated organs as animals. However, the postulated plant-specific mechanosensory network within cells to sense and res-

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pond to external stimuli (Telewski 2006); plants' intricate biochemical mechanisms to communicate with other living organisms (Bais *et al.* 2006; Schenk 2006); and plants' ability to synthesize useful organic compounds (Walker *et al.* 2003); make all the difference. In this regard, a root, properly considered "the hidden part" of a plant (Eshel and Waisel 1996), is the less known of both tissues (Bais *et al.* 2006). However, it is well recognized that local interactions between soil and plant include a broad range of biochemical, chemical and physical processes that occur as a consequence of root growth, water and solute uptake/release by roots.

Even considered under a merely abiotic point of view, the soil is an ever-changing entity, due to physical and chemical changes tied to fluctuations in the environment. In this simplistic scenario, soil-plant interactions not only generate mass fluxes between each other but also affect both the entire plant root morphology and the soil properties (Baligar *et al.* 1998; McMichael and Burke 1998; Singh and Sainju 1998; Wraith and Wright 1998; Wu *et al.* 2007).

In view of the current analysis, the scenario is further complicated when we include inhabitants of the soil other than roots. Living soil hosts are diverse: from insects and large animals, as different rodent species, to practically all kinds of microorganisms. In the sempiternal competition for nutrients, alliances and wars among soil inhabitants are common.

It has also been emphasized that minerals, organic matter, and microorganisms are integral parts of the pedosphere and related environments. These three components are not separate entities but rather a united system constantly in association and in interaction with one another in the terrestrial environment (Huang 2004). This may explain why, in order to survive in this dynamic soil, dynamic roots are essential. Dynamic roots create self-improving surroundings, the rhizospheres, where plants not only can modify soil characteristics by themselves (Neumann and Romheld 1999; Charlson and Shoemaker 2006; Kraemer *et al.* 2006), but also by recruiting other living systems to help them accomplish the task (Schenk 2006).

Considering plant roots as the point of reference, soil has been divided in three main zones: rhizoplane (root surface), rhizosphere (soil under root influence) and bulk soil (Manthey *et al.* 1994). There are three main areas of research that are carried out on the rhizosphere: a) the influence of roots on microorganisms; b) the influence of microorganisms on plant growth, and c) the rhizosphere influence on soil-borne pathogens and plant disease (Curl and Truelove 1986). We will pay attention to the second one. In this regard, dead roots and exudates are organic compound sources that attract useful, pernicious and neutral organisms to the rhizosphere. In particular, the beneficial rhizosphere microorganisms are important determinants of plant health and soil fertility. Such microorganisms participate in many key ecosystem processes such as those involved in the biological control of plant pathogens, nutrient cycling and seedling establishment (Jeffries *et al.* 2003). Positive interactions in the rhizosphere include symbiotic associations with epiphytes and mycorrhizal fungi; and root colonization by bacterial biocontrol agents and plant growth–promoting bacteria (Bais *et al.* 2006). Most of these actions are exerted through the release of compounds produced for general and specific purposes (Watt *et al.* 2006). The role of these exudates in relation to plant-plant, plant-microbe and to plant-insect interactions was extensively covered in a recent review. In that review, the authors elegantly consider roots as "rhizosphere ambassadors" in that they facilitate communication between the plant and other organisms in the soil (Bais *et al.* 2006). However, human use of soils for agricultural production could alter these relationships.

It has been pointed out that the natural roles of rhizosphere microorganisms have been marginalized due to conventional farming practices such as tillage and high inputs of inorganic fertilizer and pesticides (Mäder *et al.* 2002). Moreover, anthropogenic effects can be profound and visible. It is widely accepted that agricultural intensification has greatly increased the productive capacity of agroecosystems, but has had unintended environmental consequences including degradation of soil and water resources, and alteration of biogeochemical cycles (Drinkwater and Snapp 2007).

The above mentioned and other soil problems due to human intervention are expected to be aggravated by the requirements imposed by a growing world population (Yamaguchi and Blumwald 2005). However, the use of biotechnological tools which consider soil health preservation the premise is promising. In this regard, microbial biotechnology will continue to play an important role complementing plant biotechnology in, for instance, secondary metabolite production, biofertilizer and biopesticide production, bioprocessing, bioremediation and waste treatment (Tengerdy and Szakács 1998). Moreover, the future for soil microbiologists seems bright since new, mainly molecular, techniques offer new insight into the soil black box so that microbial community composition and microbial activities can be investigated, and even localized on a microscale (Insam 2001). More information on the role of soil microorganisms over root competence and its impact on agroecosystems productivity has been compiled by several investigators (Schenk 2006; Stockdale and Brookes 2006), and by Welbaum *et al.* (2004), respectively. The possible impact of transgenic microorganisms on the microbial diversity in soil has also been discussed (Lynch *et al.* 2004).

On the other hand, horticultural crops have a strong impact on human nutrition. A diet rich on fruits and vegetables provides an abundance of phytonutrients that are able to reduce the risks of radiation-induced cancer (Hayes 2005), to prevent many chronic diseases and to keep good human health in general (Lester 2006). The beneficial effects of these phytonutrients are dependant on their dietary intake from natural food rather than from supplements (Rock *et al.* 1996, 2004; Lichtenstein and Russell 2005). Moreover, most vegetables should be ingested crude and unprocessed (edible foods) to avoid important nutrient losses. The above mentioned and other considerations, are imposing a growing demand in obtaining high quality vegetables (Picha 2006), obviously free of potentially dangerous abiotic and biotic contaminants. In addition, the higher income generated per hectare of vegetable production, when compared to other agricultural products (Palada *et al.* 2006), justifies the use of high cost seeds, pre-sowing seed treatments, seedling transplants, soil enhancements, etc. Furthermore, the adoption of the plasticulture system – plastic-covered greenhouses, plastic mulches, row covers, high tunnels, and windbreaks both permanent and annual – is the major contributor to the positive modification of the cropping environment of vegetable crops (Lamont Jr. 2005). Such particularities related to vegetable production present diverse problems which the use of rhizosphere microorganisms could contribute to solve or minimize.

In the present review, we will pay attention to the plant-growth promoting microorganisms (PGPM) that could be used as inoculants in horticultural production, with an emphasis on *Azospirillum* spp. as a known example of the plant growth promoting rhizobacteria (PGPR) (Kloepper 1992).

Apart from being a general plant colonizer (Bashan *et al.* 2004), *Azospirillum* is remarkably versatile. *Azospirillum* is not only able to fix atmospheric N (Döbereiner and Day 1976), but also to mineralize nutrients from the soil, to sequester Fe, to survive to harsh environmental conditions, and to favor beneficial mycorrhizal-plant associations (Bashan *et al.* 2004). In addition, *Azospirillum* can help plants minimize the negative effects of abiotic stresses.

A *sine qua non* path to successful microbial plant inoculation at the field relies on the profound knowledge of the molecular strategies through which plant growth stimulation is exerted. In this regard, a part of the direct and indirect advances in understanding *Azospirillum*'s mechanisms of action is reviewed in the present work.

ABIOTIC AND BIOTIC SOIL COMPLEXITY. THE IMPORTANCE OF DYNAMIC ROOTS IN A DYNAMIC SOIL, IN CREATING SELF-IMPROVING SURROUNDINGS, THE RHIZOSPHERE

The inanimate soil is a very complex system composed of a solid matrix, gases, water, and dissolved minerals in pore spaces. The solid matrix contains inorganic particles of various sizes, shapes and chemical characteristics, and an organic matter component in various stages of decomposition (Echeverría and García 2005). While the solid matrix provides the physical space, the soil solution housed in pores is the immediate source of plant nutrients (Jungk 1996; Singh and Sainju 1998). In short, field soils not only have an enormous diversity, but also a marked physicochemical heterogeneity in pH, water content, hardness, oxygen levels and nutrient concentrations (Watt *et al*. 2006). However, this is not a static situation. Even though the term "inanimate soil" has been frequently associated to the abiotic portion, the soil is not strictly motionless or inactive. With time, all soils suffer long standing structural and physicochemical changes that culminate in the outline of a typical soil profile. In addition, soil properties are continuously changing due to shorter environmental and seasonal shifts, like those occurring in soil bulk density after freezing and thawing, raindrop impact and soil settling (Singh and Sainju 1998).

On the other hand, if the inanimate soil *per se* is a very complex and an ever changing entity, the living soil is much more complex and variable. In general, soil heterogeneity could explain why life can be so diverse in a few cubic meters. Indeed, aboveground, there are not so many places on Earth where diverse organisms can hide and live as they do belowground. Virtually every minor portion of the soil could be a place for living systems.

While plant roots, large and small animals, and visible arthropods are the most evident biota, the soil is densely populated by a myriad of diverse microorganisms. Moreover, while it is difficult to imagine organisms growing anaerobically on the atmosphere, aerobic and anaerobic organisms coexist belowground.

From all the living systems that cohabit belowground, microbes represent the largest biodiverse community (Torsvik and Øvreås 2002; Bartelt-Ryser *et al*. 2005). The microorganisms are unevenly distributed, but mainly congregated around nutrient sources and organic matter. Clay soils often have more bacteria than sandy soils because the clay creates large amounts of small pores (spaces) which offer protection for bacteria. Sandy soils with fewer aggregates and small pores are less suitable habitats for bacteria and fungi unless a large amount of organic matter is added to the soil (Six *et al*. 2004; Rong *et al*. 2007). The size, composition and activity of this microbial community is influenced by different soil features such as structure, moisture level, nutrient supply, pH, electrical conductivity, redox potential, and NaCl concentration (Lynch 1990; Nelson and Mele 2007). Such soil features account for the microbial diversity found in the rhizosphere (Curl and Truelove 1986). In addition, environmental factors as temperature, pressure, air composition, surface and spatial relations can change markedly, and therefore so do the microhabitats in the soil (Nanniperi *et al*. 2003).

In this complex and changing environment, terrestrial plants must rely on their roots to survive and proliferate. As a result, root development and distribution differ not only among plant species, but also within the same species. Many soil resources are unevenly distributed in space and time and are often subjected to localised depletion (Jackson and Caldwel 1993). Therefore, the spatial deployment of the root system will determine the ability of a plant to exploit such resources. The effects of soil nutrient heterogeneity on the performance of individual plants, populations and species mixtures have been reviewed by Casper *et al*. (2000), Hutchings *et al*. (2003) and Hodge (2004).

As soil influences roots, roots influence soil. It is a well known fact that plant roots and their associated biofilm can strongly influence soil chemistry (Heckman and Strick 1996; Walker *et al*. 2003), particularly in determining soil nutrient availability (Schenk 2006). Moreover, roots exert a remarkable influence on the microbial population living belowground. Due to the presence of different substrates released by roots, the size of soil microbial biomass is by far more pronounced in the rhizosphere than in the bulk soil (Bolton *et al*. 1992). Enhanced microbial growth and population density in the rhizosphere is called the "rhizosphere effect", and results from the release of organic compounds and mucilage by plant roots (Mallik and Williams 2005). Moreover, plant growth (the primary productivity) shows a direct correlation with the amount of microbial biomass and the organic matter level present in the rhizosphere (Paul and Clark 1996), which, in turn, could be related to the role of microorganisms in both energy flow and nutrient cycling. In this regard, the soil microbiota can contribute to plant growth by maintaining the recycling of nutrients, through the production of hormones, helping to provide resistance to microbial diseases and tolerance to toxic compounds (Morgan *et al*. 2005). Therefore, it is conceivable that a given plant could produce specific root exudates with the primary purpose of recruiting favorable organisms in its rhizosphere (Kowalchuk *et al*. 2002; Reynolds *et al*. 2003). In addition, plants have the capability to act against potential enemies through the production of antimicrobials, phytotoxins, nematicidal, and insecticidal compounds (Bais *et al*. 2006). However, the general trend seems to be the production of organic compounds that could be useful not only to invited but also to uninvited guests (Mallik and Williams 2005; Bais *et al*. 2006). In short, the relationship between organisms and roots is very complex and highly variable, resulting in beneficial, harmful, or neutral effects to a given plant (Morgan *et al*. 2005).

The brief ecological view presented above changes under an agricultural perspective, where we should consider the anthropogenic effects of human activity on soils and its biota.

POTENTIAL USE OF RHIZOSPHERE MICROORGANISMS FOR THE IMPROVEMENT OF PLANT PRODUCTIVITY IN A HUMAN-DETERIORATED ENVIRONMENT

The history of human civilization runs parallel to that of agriculture: plant domestication was the first step in the long journey that changed man from food searcher to settler. However, over the years the anthropogenic effects of classical farming on soil were costly: erosion, depletion of nutrients and contamination with natural elements, compounds and also xenobiotics were cumulative in arable lands.

Agricultural intensification has greatly increased the productive capacity of agroecosystems, but has had unintended environmental consequences including degradation of soil and water resources, and alteration of biogeochemical cycles (Drinkwater and Snapp 2007). All these setbacks were the consequence of centering agricultural production and soil conservation to cover human needs without considering either soil stability or soil health (Welbaum *et al*. 2004). The importance of soil health and soil quality in relation to sustainable land management has been discussed by Doran (2002). Regarding xenobiotics, some pesticides proved to be useful in solving many problems which affect human health and food production. However, the usage of such pesticides has occasionally been accompanied by potential risks to human beings and to the environment (Mansour 2004).

Soil erosion and nutrient loss

The potential use of microorganisms in stabilizing eroding soils (Bashan *et al*. 1999) and in restoring lost soil structure to counteract the negative effects of water deficit on plant growth (Alami *et al*. 2000; Augé 2001), has been emphasized. The first evidence of the beneficial effect of a PGPR

on soil aggregate stabilization under field conditions was recently provided (Kohler *et al.* 2006). Indeed, a calcareous soil inoculated with 10^{10} colony forming units (CFU) of *Pseudomonas mendocina* per *Lactuca sativa* L. cv. 'Focea' plant and per application has shown a significative increase of the percentage of stable aggregates (about 84% more than the control soil). The fertilized soil without inoculation and the control soil had the lowest aggregate stability.

The use of inoculants to reduce the burden of soil nutrient loss in arable lands and the subsequent fertilization procedures implies that different microorganisms could improve plant uptake of essential macronutrients (Rodríguez and Fraga 1999; Kennedy *et al.* 2004; Zahir *et al.* 2004; Orhan *et al.* 2006). Several examples of simultaneous growth promotion and increase in P and N uptake by plants as the result of phosphate-solubilizing bacteria inoculations have been reported. Inoculation with two strains of *Rhizobium leguminosarum* selected for their P-solubilization ability has been shown to improve root colonization and growth promotion and to increase significantly the P concentration in lettuce (*Lactuca sativa* L.) and maize (*Zea mays* L.) (references in Rodríguez and Fraga 1999). *Rhizobium legumenosarum* bv. *trifolii* also increased N uptake in rice. In particular, a mean fertilizer-N-use efficiency of 59.1 kg grain per kg applied urea-N was reported in non-inoculated rice (*Orizae sativa*) fertilized with 72 and 144 kg urea ha^{-1}. Under the same fertilization rates, significantly higher mean fertilizer-N-use efficiency values (in the range 68.9-78.6 kg grain per kg applied fertilizer-N) were determined in the same plant species inoculated with *R. leguminosarum* bv. *trifolii* strains E24, E27, E37 or E39 (references in Kennedy *et al.* 2004). A strain of *Pseudomonas putida* also stimulated the growth of roots and shoots and increased ^{32}P-labeled phosphate uptake in canola (*Brassica napus*). Inoculation of rice seeds with *Azospirillum lipoferum* strain 34H increased the phosphate ion content and resulted in significant improvement of root length and fresh and dry shoot weights. Simultaneous increases in P uptake and crop yields have also been observed after inoculation with *Bacillus firmus*, *Bacillus polymyxa* and *Bacillus cereus* (references in Rodríguez and Fraga 1999). *Klebsiella mobilis* strains CIAM880 and CIAM853 for inoculating potato (*Solanum tuberosum*) cultivars at low doses of nitrogen fertilizes significantly increased phosphorus and potassium in the potato tubers (Zahir *et al.* 2004). Bacterial applications of *Bacillus* OSU-142, *Bacillus* M3 and their combination (OSU-142 + M3) promoted N, P and Ca uptake of red raspberry (*Rubus idaeus* cv. 'Heritage'). The highest N (4.03%), P (0.80%) and Ca (1.44%) contents were obtained from OSU-142 + M3 application, which increased N, P and Ca content of leaves by 60%, 433% and 64% compared with control treatment (plants dipped into sterile water). All bacterial treatments, except OSU-142, were also resulted in significant Fe and Mn increase in leaves. Inoculation with M3 and OSU-142 + M3 increased Fe and Mn content of leaves by 75.6% and 64.4% and 85.1% and 117.0%, respectively (Orhan *et al.* 2006). On the other hand, the use of microbial inoculants to help plants acquire trace nutrients is a less exploited, however not less important possibility. Iron is a vital element for most of the living systems. Therefore, its limitation could cause an important nutritional disorder in agricultural crops. Even though plants can produce their own iron-mobilizing compounds (Charlson and Shoemaker 2006; Kraemer *et al.* 2006), sulfur starvation could reduce phytosiderophores release by iron-deficient barley (*Hordeum vulgare*) plants (Astolfi *et al.* 2006). Soil microorganisms may play an important role in plant Fe uptake from soils with low Fe bioavailability, but there is little direct experimental evidence to date. Based on results obtained with red clover (*Trifolium pratense* L.), an Fe-efficient leguminous plant, Jin *et al.* (2006) proposed that root exudates from minus-Fe plants selectively influence the rhizosphere microbial community, and that the microbes, in turn, favour plant Fe acquisition by producing siderophores and auxins. On the other hand, one of the widest ranging abiotic stresses in world agriculture arises from low Zn availability in calcareous soils, particularly in cereals. This fact was particularly observed in bread wheat (*Triticum aestivum* L.), durum wheat (*T. durum*), rye (*Secale cereale*), barley, triticale (x *Triticosecale* Wittmack) and oat (*Avena sativa*) growing in a severely Zn deficient calcareous soil having a clayey loam texture, pH 7.6, 16% $CaCO_3$ and 2.6% organic matter (from Cakmak *et al.* 1998, cited in Singh *et al.* 2005). In that soil, the susceptibility to Zn deficiency declined in the order durum wheat > oat > bread wheat > barley > triticale > rye. However, in rye, triticale and bread wheat growing in a growth chamber in a Zn-deficient nutrient solution under controlled climatic conditions the release rates of phytosiderophores were markedly enhanced (from Cakmak *et al.* 1998, cited in Singh *et al.* 2005). This capability in releasing Zn-mobilizing phytosiderophores was considered a relevant mechanism to adapt to Zn deficiency (Singh *et al.* 2005).

Salt and water stresses

Water and soil salinization as well as water scarcity or excess, are the main abiotic stresses agricultural production could face in the near future, aggravated by the already evident global climatic changes. Moreover, the increasing frequency of dry periods in many regions of the world and the problems associated with salinity in irrigated areas frequently result in the consecutive occurrence of drought and salinity on cultivated land (Hu and Schmidhalter 2005). Currently, 50% of all irrigation schemes are affected by salinity (Hu and Schmidhalter 2005). This situation is so serious, that, to quote a historical example, the crumbling of Mesopotamian civilization has been attributed to the progressive soil salinization derived from crop irrigation (Rattan Lal 2000). The danger that this historical event could be repeated in some regions of the world should not be excluded.

Several strategies were proposed to enhance the growth and yield of agricultural crops exposed to saline conditions. The main strategies were based on: (i) gradually improving the salt tolerance through conventional breeding and selection; (ii) crop introgressions with their wild, more tolerant to salt progenitors; (iii) domesticating halophytes by breeding and selection for improved agronomic characteristics (Shannon 1997). On the other hand, while the use of agro-biotechnological tools to cope with the negative effects of flooding on crop production has received low attention; several strategies are being tested to minimize water and saline stresses (Thomson 2003). In this regard, two main approaches are being used: (i) the exploitation of natural genetic variations, either through direct selection in stressful environments or through mapping quantitative trait loci and subsequent marker-assisted selection; and (ii) the generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to enhance salt stress tolerance (Yamaguchi and Blumwald 2005). In addition, we would like to add the use of PGPM to the strategies mentioned above. Several researchers have already tested the possibility of using PGPM to minimize saline stress on plants. The establishment and growth of the halophyte *Salicornia bigelovii* in coastal semi-arid zones can be experimentally improved using *Klebsiella pneumoniae* and *A. halopraeferens* as auxiliary biofertilisers (Rueda-Puente *et al.* 2003). Bacilio *et al.* (2004) also suggested inoculating crop seeds and seedlings with various PGPR, such as *Rhizobium* and *Azospirillum* spp., and with mycorrhizal fungi, as experimental alternatives to alleviate salt stress. Regarding water stress, even though several transgenic plants engineered to over-produce osmoprotectants showed enhanced abiotic stress tolerance, only very few attempts have been made to engineer osmoprotectants in vegetable crops (Dalal *et al.* 2006). In addition, the use of PGPM as inoculants could contribute to minimize the negative effects of several plant stresses, including water stress (Bashan *et al.* 2004; Lucy *et al.* 2004). Promising results have been also reported from plant-mycorrhizal associations (Augé 2001;

Johansson *et al.* 2004). Over 80% of all terrestrial plant species form arbuscular mycorrhizal (AM)-root symbiosis. This ancient symbiosis confers benefits directly to the host plant's growth and development through the acquisition of phosphate and other mineral nutrients from the soil by the fungus, while the fungus receives a carbon source from the host. The symbiosis may also enhance the plant's resistance to abiotic and biotic stresses (Harrier and Watson 2003). Indeed, plants colonised by AM fungi may have increased tolerance to drought. In flax (*Linum usitatissimum*), AM mycorrhizal plants had lowered sensitivity to stress, higher assimilation combined with lower increase of transpiration, and enhanced root conductance (Vonreichenbach and Schonbeck 1995, cited in Harrier and Watson 2003). More recently, Harrier and Watson (2004) have paid special importance to the bioprotection conferred to plants against many soil-borne pathogens such as species of *Aphanomyces*, *Cylindrocladium*, *Fusarium*, *Macrophomina*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Sclerotinium*, *Verticillium* and *Thielaviopsis* and various nematodes by AM fungal colonisation of the plant root. However, the same authors also pinpointed that the exact mechanisms by which AM fungal colonisation confers the protective effect are not completely understood. Moreover, a recent report stressed the still several unknowns on the systemic effects of the AM symbiosis, and the understanding of non-nutritional effects on the physiological changes occurring in the aerial parts of the host plant (Toussaint 2007). On the other hand, previous investigations have shown decreased nitrate reductase (NR) activity in lettuce under water stress, but much less in mycorrhizal-inoculated than in uninoculated plants. This effect may be a factor in the drought tolerance of mycorrhizal plants (Ruiz Lozano and Azcón 1996). In this regard, NR can be used as a biomarker of plant stress, such as drought. It has been shown that NR activity decreases in plants exposed to water limitation because of a lower flux of nitrate from the soil to the root. In addition, the presence of NR activity in AM fungi and the increase of NR activity in the AM symbiosis have also been shown. Moreover, under induced water deficit, positive effects of mycorrhizal inoculation on nitrate acquisition and assimilation have been reported in herbaceous plants with high water requirements and in semiarid Mediterranean shrubs, although these effects depended on the associated mycorrhizal fungus and the host plant species (Caravaca *et al.* 2005).

Soil contamination

Heavy metal (HM) contamination of soils is another matter of utter importance. Both Cd and Pb are considered the most significant HM which affect vegetable crops (Kachenko and Singh 2006). In particular, Cd is one of the most mobile HM in the soil-plant system. It is equally toxic for plant, animal and human organisms, easily taken up by plants and considered amongst the most biologically accumulative HM (Lehoczky *et al.* 2000). Therefore, HM contamination is mentioned as one of the major problems which agriculture faces in industrial and urban areas (Puschenreiter and Horak 2003). On the other hand, although not anthropogenic in origin, soil and groundwater contamination by As is also an enormous problem in different parts of the world.

The use of biotechnological tools could help reduce the risks of producing edible foods in these contaminated regions. In particular, phytoremediation is the emerging *in situ* technology aimed at using plants to remove, transfer or stabilize contaminants from soil, sediments and water (Khan 2005). This technology also includes strategies where plant-PGPR associations could be used to remediate contaminated soils (Koo and Cho 2006; Zhuang *et al.* 2007). In addition, the participation of AM fungi could contribute to improve plant-based environmental clean-up systems (Göhre and Paszkowski 2006). For years, studies with AM fungi have focused on their ability to enhance nutrient uptake in a nutrient deficient soil and have ignored the role

they may play in phytoremediation (Khan 2005). Binding and immobilization of metals in the mycorrhizosphere may contribute to the direct phytoremediation effects. Indirect effects may include the mycorrhizal contribution to balance plant mineral nutrition, particularly P nutrition, leading to increased plant growth and enhanced metal tolerance (Christie *et al.* 2004).

In addition, plant exudates could stimulate the survival and action of the microbes useful in soil remediation, resulting in a more efficient degradation of pollutants (Kuiper *et al.* 2004). However, while transforming toxic xenobiotics into less harmful sub-products could be a valid technique to remediate soil contaminated with these compounds, the introduction of microbial inoculants to help plants avoid or minimize uptake could be the choice for HM and metaloid contaminants. In this regard, *Kluyvera ascorbata* SUD165 and a siderophore-overproducing mutant of this PGPR were able to minimize the inhibitory growth effects of high concentrations of Ni, Pb, and Zn on tomato (*Lycopersicon esculentum*), canola (*Brassica napus*), and Indian mustard (*Brassica juncea*) seedlings cultivated for 25-42 days in a HM-contaminated soil (Burd *et al.* 2000). The authors suggested that the ability of these bacteria to protect plants against the inhibitory effects of high HM concentrations could be related to the bacteria providing the plants with sufficient iron. Other favourable effects could be provided by co-inoculation. As an example, it was recently demonstrated that PGPR isolated from a Cd-contaminated soil increased rhizobial nodulation of clover plants growing in this soil (Vivas *et al.* 2003). One explanation for this effect could be that the PGPR accumulated Cd, therefore preventing Cd toxicity and enabling nodulation. In addition, an increase in soil enzymatic activities (phosphatase, β-glucosidase, dehydrogenase) and of auxin production around PGPR-inoculated roots could also be involved in the PGPR effect on enhancing nodulation (Barea *et al.* 2005).

On the other hand, the application of rhizosphere processes to the development of phytoremediation technologies for As-polluted soils has been difficult due to the lack of information that directly addresses the fate of As in the rhizosphere (Fitz and Wenzel 2002). However, a major step towards the development of phytoextraction of As-impacted soils was the recent discovery of As hyper accumulation in ferns, which seem to have both constitutive and adaptive mechanisms for accumulating or tolerating high As concentrations (Silva Gonzaga *et al.* 2006).

POTENTIAL USE OF PGPM AS A PALLIATIVE OF DIVERSE FACTORS AFFECTING VEGETABLE PRODUCTION

Soil treatments

Solarization

Many vegetable growers rely on methyl bromide or other soil fumigants to manage soil pathogens, nematodes, and weeds. However, the growing demand on putting healthy, non contaminated vegetables on the human table is giving a thrust to alternatives like soil solarization, the hydrothermal process of disinfesting soil of plant pests that is accomplished through passive solar heating (Stapleton 2000). The total soil microbial population decrease associated to the solarization process has been attributed to the direct thermal effect on heat-sensitive microorganisms and to the indirect action of biotoxic volatile compounds released from the organic matter (Stapleton 1997). In addition, the possibility that microorganisms helpful to weeds but not to agricultural crops could be attracted by weed exudates to the rhizosphere, should not be discarded. Diminishing weed population by soil solarization would restrict that possibility. Another effect of soil solarization is the increased availability of mineral nutrients such as NH_4-N, NO_3-N, P, Ca, and Mg, as a result of the death of the microbiota (Chen and Katan 1980; Stapleton *et al.* 1985, 1990). Despite a general de-

crease in the total microbial population, a significant increase of rhizosphere competent bacteria, namely *Bacillus* spp. and fluorescent pseudomonads, was observed in the rhizosphere of tomatoes and sweetpotatoes grown in a solarized soil when compared to a nonsolarized soil (Stevens *et al*. 2003). Whatever the situation, both the increase in mineral nutrients availability and the predominance of heat-selected microorganisms could account for the plant growth and yield enhancements observed in plants grown in solarized soils (Katan 1985; Stapleton and de Vay 1986). Under these considerations, the already known effects PGPR inoculants could exert on vegetable growth and yield could be more marked in solarized soils than in non-solarized ones. In fact, field trials performed to evaluate tomato (*Solanum lycopersicon* cv. 'Solar Set') and pepper (*Capsicum annuum* cv. 'Jupiter') transplants amended with formulations of several PGPR in a production system that included soil solarization has shown promising results in increasing transplant growth, vigor, survival, and yield (Kokalis-Burelle *et al*. 2002). In these studies, highly significant increases in tomato (*S. lycopersicon* cv. 'Solar Set') and pepper (*C. annuum* cv. 'Jupiter') transplant growth occurred in response to most formulations obtained by combining *Bacillus subtilis* strain GBO3 to *Bacillus amyloliquefaciens* strain IN937a (LS213), *B. subtilis* strain GBO3, *Bacillus pumilus* strain SE34 (LS254), *B. subtilis* strain IN937b (LS255), *B. pumilis* strain INR7 (LS256), or to *Bacillus cereus* strain C4 (LS261). In tomato, LS254 and LS256 produced 395% and 337% increases in dry root weight compared to the untreated control, respectively. In pepper, transplant vigor was also increased by all PGPR treatments, but the most dramatic effect was observed with LS261, which resulted in a 565% increase in dry root weight compared to the untreated control. Moreover, the combination LS256-soil solarization had significantly higher pepper yield than the untreated control (Kokalis-Burelle *et al*. 2002).

Fertilization

Some vegetables are very sensitive to nutrient soil loss thus horticultural soils are usually enhanced by different treatments, fertilization being the most common of all of them. In soils with high N content compared to that of P and K, foliage growth predominates over reproductive organs. Therefore, horticultural crops where leaves are the product usually require higher N than do other crops. In particular, lettuce requires moderately large amounts of nitrogen and phosphorous. Under severe shortage of nitrogen, lettuce has been observed to grow at a very slow rate, as well as to have an abnormally low water content, low reduced-nitrogen content and negligible nitrate content (Seginer 2003). To counteract these effects, farmers usually apply a uniform rate of N to agricultural fields assuming that N sources, sinks, and mechanisms for toss are constant across fields. It is well documented that variability of soil that affect N sources makes managing N to maximize N use efficiency (NUE) difficult (Delgado 2002).

Regarding phosphorus, the phosphate fertilizer recommendations for field vegetables are usually much higher than the phosphate removed by the marketable crop. As a result soils may become saturated with phosphate. This increases the risk of phosphate emission to ground and subsurface water (van Wijk and Bouten 2002). In addition, heavy fertilization has been shown to reduce the AM colonization of lettuce and its ability to increase plant nutrient uptake and plant growth (Azcón *et al*. 2003).

On the other hand, high combined irrigation and fertilization (fertigation) frequency induced a significant increase in lettuce yield, mainly at low nutrients concentration levels. Moreover, this treatment enables to reduce the concentrations of immobile elements such as P, K and trace metals in irrigation water, and to lessen the environment pollution by discharge (Silber *et al*. 2003).

Whatever the application method used, if fertilizers are spread all over the ground then only a minor part of the nut-

rients would be used by the plant while the rest would saturate the soil and eventually, percolate to aquifers. In the late 1960s, microirrigation (drip or trickle) systems were developed and have slowly replaced many of the sprinklers and some of the seepage systems. Microirrigation is currently used on 5% of irrigated crops. This highly efficient water system (90% to 95%) is widely used on high value vegetables, particularly polyethylene-mulched tomato (*Lycopersicon esculentum*), pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), and cucurbits (Locascio 2005). Subsurface drip irrigation and fertigation offer potential for increased water and N fertilizer use efficiency by broccoli, and decreased groundwater NO_3 pollution (Thompson *et al*. 2002).

It would be interesting to evaluate if high combined irrigation, fertilization and PGPM (biofertigation) could further improve the benefits described above. The use of PGPM could contribute to reduce the use of inorganic and organic fertilizers (Freitas *et al*. 2003), to minimize HM contamination and also to help vegetables in their fight against diverse pathogens (Siddiqui *et al*. 2004). In this regard, it has been postulated that the effective use of AM inocula and resulting colonization would results in similar plant growth and yield and reduce the need for high levels of P application which historically results in soil and ground water contamination in many agricultural areas (Azcón *et al*. 2003). In addition, the use of mycorrhizal fungi-PGPR inoculum to improve plant performance offers new insights. More detailed information on AM and PGPR interactions can be found in the literature (Artursson *et al*. 2006; Bending *et al*. 2007).

Apart from inorganic fertilizers, different organic substrates have been tested for their suitability for plant propagation of vegetables. These include farmyard manure compost, household waste compost, chicken manure, vermincompost, biostabilized compost, olive mill residues, etc., all of them with variable results (Martin *et al*. 2002; Premuzic *et al*. 2002; Stintzing and Salomon 2002; Wivstad *et al*. 2003). Manure amendments of soils are very common in vegetable production, but the NUE have shown to be variable according to the rooting depth, which in turn varies among carrot, early cabbage, onion and lettuce (Thorup-Kristensen 2006). Again, the use of PGPM that increase root growth could help to improve NUE.

No matter which fertilizer is used, collateral unintended effects as HM accumulation and phytotoxic effects could arise with the use of these substrates (Eklind *et al*. 2001; Martin *et al*. 2002). It has been emphasized that some phosphorus and trace element fertilizers may contain elevated amounts of toxic metals such as Cd and repeated uses of the fertilizers at high rates over time may increase Cd uptake by plants (Huang *et al*. 2003).

Soil contamination

A serious risk for human health would be the use of land-filled suburbs of large cities to grow edible foods, where high concentrations of HM residues could be contaminating the soil. The increased danger of growing vegetables in these sites and in metal smelter contaminated ones, has been recently emphasized (Kachenko and Singh 2006). Despite the danger, the social situation could prompt poor people to cultivate edible foods in those places anyway. In this regard, lettuce is one of the most easily grown and consumed vegetables. This species accumulates relatively high amounts of HM such as Cu, Zn and Pb (for references see Jordão *et al*. 2006) and may be used as an indicator of metal contamination in soils. In a soil amended with mine wastes, lettuce (*L. sativa* L. cv 'Iceberg') accumulated significantly more metals than other species such as bean (*Vicia faba* cv. 'Roma bush') and tomato (*Lycopersicon esculentum* cv. 'Better Boy') (Cobb *et al*. 2000). Moreover, it has been found the plant that best accumulated Cd in its leaves (Lehoczky *et al*. 2000). These investigators also reported that Cd concentration in lettuce leaves could be 1.4-16 times as

much as the total Cd content of the soil, being the hydrolytic acidity the factor that mainly increases the Cd uptake. In agreement with these data, McBride (2002) has shown that long-term bioavailability of Cd depends primarily on the soil pH and Cd concentration in the soil. In spinach, Cu, Zn and Cd uptake appeared to be highest in the cultivar that exuded more organic acids (oxalate, citrate and malate) by roots (Romer and Keller 2002). These compounds, considered part of the dissolved organic matter (DOM) in soil solution, also increased the toxicity and bioavailability of Cu in lettuce sprouts (Inaba and Takenaka 2005). Interestingly, a combined fertilization of Zn and Cu bound to zeolite decreased Cd levels in the aboveground biomass of both spinach (*Spinacia oleracea* L. cv. 'Matador') and wheat (*Triticum aestivum* L. cv. 'Ñandu') and in wheat grains in a soil treated with $CdSO_4$ (Puschenreiter and Horak 2003). This study demonstrated that fertilization with slow-release micronutrients can decrease the concentration of Cd in edible parts of crops and may thus improve the quality of agricultural products grown on high Cd soils.

On the other hand, attempts to reduce As uptake by lettuce using different combinations of Fe oxides and lime showed promising results with minimal changes in yield. However, insufficient lime lowered soil pH and mobilized potentially dangerous HM (Warren and Alloway 2003). These and other soil factors regulating As bioavailability and the different strategies that could be used to minimize the problem, has been recently reviewed (Mahimairaia *et al.* 2005). As already mentioned, the use of biotechnological tools would be a valid alternative in contributing to put vegetables non-contaminated by HM and/or metaloid on the human table.

Despite the optimistic perspectives mentioned above, root growth increased by auxin-producing rhizomicroorganisms could lead to an unexpected risk of HM accumulation by the plant. In fact, more basic and applied research are needed in this field to assess that the close relationships among HM contaminants and trace nutrients influencing each other in their uptake by the plant would not be altered by the introduced microorganisms and led to unwanted results.

Even though other systems different than soil as a support could be used to grow vegetables such as hydroponics (Braz *et al.* 2003) and aeroponics (Demsar and Osvald 2003), they are not free of problems. For example, phytotoxic effects due to ferulic acid accumulation in commercial closed, hydroponic lettuce cultures could be ameliorated by bacterial isolates (Caspersen *et al.* 2000).

Transplant establishment on soil

While some crops can grow to maturity and produce well from either direct seeding, others need to be transplanted to obtain maximum yield. This last procedure is very common in vegetable production, particularly in the United States, where the use of transplants to establish horticultural crops in the field is an accepted practice (Russo 2006). Poor establishment of direct-seeded lettuce crops using both pelleted and raw seeds, particularly during conditions of environmental stress, has led to the use of transplants as a means of establishing economically viable plants stands. Guzmán *et al.* (1989) reported that superior plant stand was the major contributing factor for increased marketable yields from transplanted crisphead and romaine lettuce (*Lactuca sativa* L). Indeed, transplants are used to provide an early start for plants, produce seedlings of more consistent quality and control spacing of plants in the field (Russo 2006).

However, while transplanting could be highly convenient for the farmer, the procedure by itself is a stressful factor for the plant. It is a well known fact that plants previously exposed to one type of abiotic stress could tolerate better a second different type of stress. In this regard, appropriate levels of water deficit stress could be used as a management tool to produce seedlings conductive to the

transplanting process (Liptay *et. al.* 1998). In our opinion, PGPR inoculation could provide a sort of stress protection that could help the trasplant to acclimate to its new place without the need of a previous water stress. The idea of using PGPR to improve transplant performance at the field has already been put into practice (Kokalis-Burelle *et al.* 2002, 2003, 2006; Russo 2006). In addition, the ability to form adventitious roots is critical for plants that are propagated through vegetative cuttings and, as a consequence, problems associated with rooting of cuttings frequently results in significant economic losses (de Klerk *et al.* 1999). In this regard, *Azospirillum* inoculation could help to stimulate adventitious root development (ARD). Recently, nitric oxide (NO) was reported to be involved in the regulation of root hair formation in *Arabidopsis* and lettuce, mediating an auxin-triggered signalling cascade (Lombardo *et al.* 2006). More recent results show that *Azospirillum* could produce NO that in turn stimulate adventitious root formation in tomato (unpublished results). Despite the promising prospects offered by the use of biotic amendments, the need or further research to determine whether plants developed under these treatments are different from untreated controls when seedlings are established in the field, has been emphasized (Russo 2006).

On the other hand, seedlings production in seedling trays or in standard commercially used press pots are two well known raising systems from where lettuce plants could be transplanted (Gruda and Schnitzler 2000). Here the effects of PGPM inoculation in stimulating nutrient uptake efficiency through root growth enhancement could be not so helpful taking into account the confinement stress imposed to roots by the container size (Tisserat and Silman 2000). More basic and applied research is needed to finely adjust this possible antagonistic effect on vegetable growth in these systems.

Seed ageing

Seed quality in many horticultural species depends significantly on the obtaining process and the subsequent management. Several months could pass from seed harvest to seeding. In this period the quality of seeds, defined as the ability to germinate, may decline. The delay in germination that accompanies seed ageing is generally related to seed deterioration during dry storage. Under this low moisture condition, the seed processes involved in repairing damages may be impaired. However, soon after imbibition and before germination, these processes were reported to become active in aged seeds (Elder and Osborne 1993). An important component of seed vigor is the rate of germination following imbibition. Several seed priming treatments have been developed to increase the speed and synchronicity of germination (Bradford 1986; Ashraf and Foolad 2005). In those procedures that involve prehydration, seeds are allowed to imbibe sufficient water to trigger germination, but not enough to allow radicle to emerge through the seed coat. Particularly, osmopriming (soaking in solutions of different organic osmotica) has been used as an invigoration treatment to improve the rate and uniformity of germination of vegetable seeds (Burgrass and Powell 1984).

On the other hand, it has been reported that inoculation with PGPR could increase germination, seedling emergence, growth and yield of cereal and non cereal crops (Zahir *et al.* 2004). The authors have included alfalfa (*Medicago sativa* L), barley (*Hordeum vulgare*), canola (*Brassica napus*), chick pea (*Cicer arietinum*), cotton (*Gossypium* sp.), jojoba (*Simmonsis chinensis*), lettuce (*L. sativa* L.), oat (*Avena sativa* L.), maize (*Z. mays* L.), pepper (*C. annuum*), potato (*S. tuberosum*), rice (*O. sativa* L.), radish (*Raphanus sativus*), rapeseed (*Brassica napus*), sorghum (*Sorghum bicolor*), spruce (*Picea* sp.), tomato (*Lycopersicum esculentum*) and wheat (*T. aestivum* L.) in their review (Zahir *et al.* 2004). Arkhipova *et al.* (2005) reported that *B. subtilis* is able to produce cytokinins and influence lettuce growth. Considering *Azospirillum*'s ability to produce and/or mo-

dify plant growth substances – deconjugation of GA-glucosyl conjugates (Piccoli *et al*. 1997), and 3β-hydroxylation of inactive 3-deoxy GAs present in roots to active forms (Kobayashi *et al*. 1994; Piccoli *et al*. 1997; Cassán *et al*. 2001) –, the possibility to improve germinability in aged vegetable seeds is currently studied. Lettuce seeds naturally aged for one year had a low germination rate, vigor and emergence percentage, and an increased abnormal seedling fraction. However, when inoculated with 10^7 cells of *A. brasilense* per seed with or without previous osmopriming, enhanced vigor and emergence rate and decreased abnormal seedling fraction were obtained. Therefore, inoculation with *A. brasilense* along or after an osmopriming treatment could mitigate the negative effects of ageing on lettuce seeds (Carrozzi 2005). It was stressed however, that a better understanding of the metabolic events that take place in the seed during priming and subsequent germination will improve the effective application of this technology (Ashraf and Foolad 2005).

Seed germination and vegetable production under saline or water stresses

Even when high quality seeds could be at hand, seed germination after sowing would be affected by abiotic stresses such as those imposed by saline soils, drought, or irrigation with saline water. These are common problems in horticultural production, broadly carried on in regions where irrigation water salinity may be worrisome (Graifenberg *et al*. 1993). Moreover, vegetable crops are generally more salt sensitive than grains and forages (Shannon 1997). Amongst different vegetables, *L. sativa* is considered to be a relatively salt sensitive one (Martínez *et al*. 1996), more than broccoli (*Brassica oleracea* var. *italica*), cucumber (*Cucumis sativus*), spinach (*Spinacia oleracea*), cabbage (*Brassica oleracea*) and pepper (*C. annuum*), but less than carrots (*Daucus carota*), onions (*Allium cepa*) and radish (*Raphanus sativus*) (Xu *et al*. 2000). Indeed, lettuce germination rate, root elongation, seedling growth and mature vegetative growth were severely affected by high (60 mM) NaCl in nutrient solution (Kaya *et al*. 2002). Recent results have shown the feasibility to use *Azospirillum* inoculation to mitigate the negative effects of NaCl on these parameters (Barassi *et al*. 2006). A liquid *Azospirillum brasilense* Sp245 inoculum obtained aerobically up to a 10^7 bacterial cells seed^{-1} concentration was incorporated into lettuce (*L. sativa* L. cv. 'Mantecosa') seeds during the imbibition process (for more details see Barassi *et al*. 2006). In non-inoculated lettuce seeds, germination percentage dropped from 88.6 to 11.1% when germinated in 80 mM NaCl. In contrast, at the same NaCl concentration the germination percentage had fallen from 97.6 to 74.8% in *Azospirillum*-inoculated seeds. Even after 30 d storage, inoculated dried seeds were able to maintain the ability to mitigate the negative effects of salt on lettuce germination. Moreover, plants grown from inoculated seeds and irrigated with saline media displayed higher total fresh and dry weights and biomass partition to the aerial portion, than non-inoculated controls (Barassi *et al*. 2006). *Azospirillum brasilense* Sp245-inoculated carrot (*Daucus carota* cv. 'Beatriz INTA') seeds were treated according to the same protocol described for lettuce (Barassi *et al*. 2006). Similar effects of *A. brasilense* on alleviating salt stress syndrome on both germination and plant growth in carrot (*D. carota*), were observed (Ayrault 2002). This bacterium was also able to relieve salinity stress in chickpea (*Cicer arietinum*) plants (Hamaoui *et al*. 2001).

On the other hand, drought after planting affects both germination and initial growth, reducing the density of plants obtained and consequently the final yield per area unit. This effect is specially marked in maize, where the rapid dehydration of seedlings is associated to the relative small size of their roots (Cutforth *et al*. 1986). Based on the growth-promoting effect *Azospirillum* exerts on roots, experiments performed on maize demonstrated lesser negative effects of water stress on growth and water status in seed-

lings germinated from inoculated seeds than in those emerged from non inoculated controls (Casanovas *et al*. 2002). In these experiments, maize seeds (*Z. mays* L. hybrid DK 636) were inoculated during seed imbibition with an *Azospirillum brasilense* Sp245 inoculum at a concentration of 10^7 bacterial cells seed^{-1}. Seedlings were grown in a greenhouse, under full, medium (50% water restriction) and low irrigation (75% water restriction) rates. Seed inoculation with *Azospirillum* improved both leaf relative water content and water content, prevented a significant water potential drop in leaves, increased root growth, total aerial biomass and foliar area, and promoted proline accumulation in both leaves and roots, in seedlings grown for 15 d at a low irrigation rate (Casanovas *et al*. 2002).

This beneficial effect of *Azospirillum* inoculation was previously observed in wheat (*Triticum aestivum* cv. 'Buck Ombú') seeds, where a mitigating effect of salt stress was also evident (Creus *et al*. 1997). In these studies it was shown that *A. brasilense* inoculation provoked a significative increase in shoot elongation rate in the range of 38 to 160% above the control non-inoculated plants growing in 160 or 320 mM NaCl. Values of fresh and dry weights, water content and relative water content were significantly higher in shoots from inoculated plants than in controls under 320 mM NaCl, 20% polyethylene glycol (PEG) 6000, or 30% PEG 6000 stresses. This could be the result of a better water uptake due to *A. brasilense*, which in turn is reflected by a faster shoot growth in inoculated seedlings exposed to severe salt or osmotic stresses. Regarding vegetables, PGPR isolates have been useful in protecting tomato (*L. esculentum* Mill cv. 'F144') and pepper (*C. annuum* L. cv. 'Maor') from water stress (Mayak *et al*. 2004). Indeed, *Achromobacter piechaudii* ARV8-inoculated tomato and pepper seedlings exposed to a period of water deprivation and recovery from stress showed significatives higher fresh and dry weights than their corresponding non inoculated controls. These growth promoting effects could be related to the bacterial capability to produce ACC-deaminase and its effect on reducing ethylene levels in water stressed plants (Mayak *et al*. 2004). In addition, inoculating carrot roots with *Pseudomonas fluorescens* and *P. putida* resulted in enhanced seedling growth under normal moisture conditions (Lada *et al*. 2004). Whatever the microorganism is intended to be used as inoculant, a better knowledge of the interactions among plants, microorganisms and the environment would provide tools for a more rational, really sustainable agriculture. In this regard, *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 to a better understanding of other potentially useful microbial-plant associations.

AZOSPIRILLUM-PLANT INTERACTIONS

Azospirillum spp. is included into the alpha subclass of Proteobacteria belonging to the IV rRNA superfamily (Xia *et al*. 1994). This group of free-living rhizobacteria encompasses ten species, each one classified according to its particular biochemical and molecular characteristics: *A. lipoferum* 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. largimobile* (Dekhil *et al*. 1997); *A. doebereinerae* (Eckert *et al*. 2001); *A. oryzae* (Xie and Yokota 2005); *A. melinis* (Peng *et al*. 2006) and recently *A. canadensis* (Mehnaz *et al*. 2007). Although *Azospirillum* was first isolated from cereals and most of the initial inoculation has been done on the main cereal crops, there are more non-cereal species successfully inoculated with *Azospirillum* than cereals. *Azospirillum* strains have no preferences 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 general root colonizer and not a plant specific bacterium (for details on plant species see Bashan and Holguin 1997; Bashan *et al*. 2004).

Azospirillum is not the only microorganism capable of colonizing vegetables and inducing beneficial effects on them, but it congregates several characteristics present in different microorganisms, (detailed in the following section) that make it a valuable PGPR. Indeed, both a higher growth and yield observed in *Azospirillum*-inoculated subtropical grasses (*Z. mays*, *O. sativa*, *Saccharum officinarum*, *Sorghum bicolor* and forages such as *Digitaria* spp.) were primarily attributed to the biological N_2 fixation (BNF) exerted by the bacteria (Döbereiner and Day 1976) (discussed below). The process is performed by a nitrogenase complex, and occurs when the availability of N compounds and oxygen tension are low (Steenhoudt and Vanderleyden 2000). Even though this characteristic could be extremely valuable in agriculture, later field studies including those in which isotopic dilution techniques were used, failed to demonstrate a significant BNF in *Azospirillum*-inoculated crops (van de Broek *et al.* 2000). Even at the lab level, the growth promotion effect induced by the inoculation of axenic seedlings could not be ascribed to BNF (Bashan *et al.* 1989). Further studies pinpointed the positive bacterial effects on plants on morphological and physiological changes in the inoculated roots that would lead to an enhancement of water and mineral uptake (Okon and Kapulnik 1986). Other physiological changes observed in the inoculated plant subjected to abiotic stresses were reported. *Azospirillum*-inoculated wheat (*T. aestivum*) seedlings subjected to osmotic stress developed significant higher coleoptiles, with higher fresh weight and better water status than non-inoculated seedlings (Alvarez *et al.* 1996; Creus *et al.* 1998). Taking into account that a plant exposed to salt stress also suffer water deficit, it was proved that inoculating with 10^8 cells of *A. brasilense* on root seedlings and thereafter exposed to mild and severe salt stress significantly reversed part of the negative effects. *Azospirillum*-inoculated wheat seedlings were able to survive when exposed to up to 320 mM NaCl for three days (Creus *et al.* 1997). Uniform wheat (*T. aestivum* cv. 'Buck Ombú') seedlings (1-cm length) were inoculated with *A. brasilense* Sp245, performed by dipping roots in a 10^8 bacterial cells mL^{-1} suspension for 3 h. After that, the inoculum was replaced either by distilled water, 160 mM NaCl, 320 mM NaCl, 20% PEG 6000, or 30% PEG 6000, and seedlings were grown at 20ºC in a growth chamber in the dark up to 3 d. Fresh weight, fresh weight/dry weight, water content, and relative water content were higher in shoots from inoculated plants than in stressed controls (Creus *et al.* 1997). These changes could be explaining in part a better performance of crops. Indeed, field experiments carried out with *Azospirilla*-inoculated *S. bicolor*, *Z. mays* and *T. aestivum* have shown significantly increased yields accompanied by better water and mineral uptake, less canopy temperature and improvement in growth and yield (Sarig *et al.* 1988; Okon and Labandera-González 1994; Casanovas *et al.* 2003; Creus *et al.* 2004). An early review on the benefits a plant could obtain following *Azospirillum* inoculation stressed the importance of improving plant-water status for plants (Okon 1985). In this sense inoculation technology with *Azospirillum* could be extended to arid soils in order to protect crops against drought. As the main effect of *Azospirillum* is to promote a more developed radical system, plant adaptation to water stress could be enhanced in inoculated crops (The promoting effects on roots are discussed in the next section of this review). In this regard, experiments concerning the response mechanisms of plants to water stress demonstrated that significantly higher water content, relative water content, water potential, apoplastic water fraction, and lower cell wall modulous of elasticity values were obtained in *Azospirillum*-inoculated plants suffering drought. Yield loss due to stress diminished in *Azospirillum*-inoculated wheat (*T. aestivum* cv. 'Pro-INTA Oasis') and grains had significantly 38.4; 22.2 and 125 % higher Mg, K and Ca respectively, than non-inoculated plants (Creus *et al.* 2004).

Anyway, it is agreed that the beneficial *Azospirillum* effects on plants relies upon good root colonization. If a positive effect of inoculation with *Azospirillum* sp. is expected, significant root colonization should happen first. Root colonization is important as the first step not only in infection by soil-borne pathogens but also in beneficial associations with microorganisms. The first event in the colonization process is the attachment of bacteria to roots. In the *Azospirillum*-root interaction, this is a two step process comprised of adsorption, mediated by bacterial proteins, and anchoring involving bacterial polysaccharides (Michiels *et al.* 1991). To attach and colonize plant root surfaces *Azospirillum* spp. must first rely in a process that depends on active motility and chemotaxis toward root exudates.

Motility is an important trait for competitive pathogens and beneficial microbes and enables the participation in this cross talk between plant roots and microbes. Chemical attraction of soil microbes to plant roots, or chemotaxis, is a well understood mechanism involved in initiating cross talk between plant roots and microbes.

The distribution of *Azospirillum* in the root was studied with different techniques. Using the gfp-protein to tag bacteria Liu *et al.* (2003) confirmed previous findings about colonizing patterns. The bacteria is established mainly on the root surface but some strains of *A. lipoferum* and *A. brasilense* but not others are capable of colonizing the root interior in the apoplast and intercellular spaces. This ability could mean a lower vulnerability to harsh conditions imposed by the soil and/or the environment, which in turn could imply a more efficient promotion of plant growth (Sturz and Nowak 2000). In this regard, rhizobacteria established inside roots in intimate association with plants are considered endophytes. These microorganisms live outside the symplast and do not produce nodules, but can produce signal compounds that stimulate plant growth, enhance plant disease resistance, or improve mobilization of soil nutrients.

The beneficial effects *Azospirillum* exerts on plants, whether they are achieved under normal or environmental stressing conditions, rely on molecular mechanisms that are poorly understood.

AZOSPIRILLUM'S MECHANISM OF GROWTH PROMOTION

Several mechanisms, other than BNF mentioned above, have been postulated to explain how *Azospirillum* enhances growth and development of plants, such as phytohormone production and nitrate reduction (Bothe *et al.* 1992; Bashan and Holguin 1997; Steenhoudt and van der Leyden 2000). Nevertheless, to date no unique mechanism had been established to explain the growth promotion capability of these bacteria. Instead, the most accepted hypothesis postulates that a sum of events accounts for the general plant growth promotion effect (Bashan and Holguin 1997).

Azospirillum spp. is not considered to be a classic biocontrol agent of soil-borne plant pathogens. However, there have been reports on moderate capabilities of *A. brasilense* in biocontrolling crown gall-producing *Agrobacterium* (Bakanchikova *et al.* 1993); bacterial leaf blight of mulberry (Sudhakar *et al.* 2000); and bacterial leaf and/or vascular tomato diseases (Bashan and de-Bashan 2002a, 2002b). In addition, *A. brasilense* can restrict the proliferation of other nonpathogenic rhizosphere bacteria (Holguin and Bashan 1996). These *Azospirillum*'s antibacterial activities could be related to its already known ability to produce bacteriocins (Oliveira and Drozdowicz 1987) and siderophores (Tapia-Hernández *et al.* 1990; Shah *et al.* 1992). In addition, it was recently reported that *A. brasilense* can synthesize phenylacetic acid (PAA), an auxin-like molecule with antimicrobial activity (Somers *et al.* 2005). PAA was detected by concentration of culture supernatant only in the presence of 0.5 mM phenylalanine added to the media as a precursor molecule. It was also detected at the onset of secondary metabolism. However, since the authors worked only with concentrated supernatant extracts, it is yet unknown if the production of PAA by *A. brasilense in vivo* is sufficient to

be considered of ecological importance (Somers *et al.* 2005).

As a primary target, the root is the organ that shows the first stimulating bacterial effects. This was particularly remarkable in plants inoculated with *Azospirillum* spp. (Okon 1985). Upon inoculation the root displayed a significant increase in the number and the length of root hairs, the rate of appearance and number of lateral roots, the diameter and length of lateral and adventitious roots and the root surface area (Kapulnik *et al.* 1985; Fallik *et al.* 1994; Dobbelaere *et al.* 1999; Creus *et al.* 2005). 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 of their effect on root development (Mantelin and Touraine 2004). Nevertheless, more direct effects on root transport systems cannot be ruled out. Bertrand *et al.* (2000) showed that an *Achromobacter* sp. enhanced NO_3^- 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. 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 occured 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. In addition to its physiological activities on root membranes, there is evidence that fatty acid composition of main phospholipids in roots is affected by inoculation by *A. brasilense* (Pereyra *et al.* 2006). While the effects produced by PGPR are rather well described, the underlying signaling mechanisms triggered in plants by these bacteria are not yet identified.

It has been assumed that all the *Azospirillum*'s effects on plants are dependent on the plant species and cultivar inoculated and on the inoculum concentration used (van de Broek *et al.* 2000). Regarding the last mentioned factor, inoculation of many different plant species with *Azospirillum* in a range between 10^6 to 10^8 cells per seedling provoked root elongation (Kapulnik *et al.* 1985; Creus *et al.* 1996). However, higher concentrations of bacteria *A. brasilense* always results in restricted root growth (Harari *et al.* 1989; Pereyra *et al.* 2007). Thus, there exists an optimum bacterial concentration for triggering root elongation. The production of plant growth substances by *Azospirillum* has often been proposed as one of the key factors responsible for the observed plant growth promotion, as plant growth substances could be detected in the supernatant of *Azospirillum* cultures. Tien *et al.* (1979) showed that *Azospirillum* is able to produce auxins when exposed to tryptophan. In fact, a variety of auxins like indole-3-acetic acid (IAA), indole-3-pyruvic acid, indole-3-butyric acid and indole lactic acid (Costacurta *et al.* 1994; Martínez-Morales *et al.* 2003); cytokinins (Horemans *et al.* 1986; Cacciari *et al.* 1989) and gibberellins (Bottini *et al.* 1989) were detected, with auxin production being quantitatively most important. Studies on IAA production showed that it relay on the type of culture media and availability of tryptophan as a precursor. Among the strains tested *A. brasilense* Cd produced the highest level of IAA (approx. 380 μmol.L^{-1}) (El-Khawas and Adachi 1999). Two types of experimental evidence support the bacterial hormone production as the plant growth promoting grounds. On one hand, morphological changes on the plant root following *Azospirillum* inoculation could be mimicked by applying a combination of plant growth substances. Combinations of diferent amounts of indole acetic acid, gibberellin, and kinetin all of them in ranges of 0.001 to 0.05 μg. mL^{-1} produced changes in root morphology of pearl millet (*Pennisetum americanum* L.) similar to those produced by inoculation with *A. brasilense* (Tien *et al.* 1979; Jain and Patriquin 1984). In addition, the effect of *Azospirillum* inoculation on root elongation of wheat plants (Kolb and Martin 1985; Dobbelaere *et al.* 1999) and on branching of wheat root hairs (Jain and Patriquin 1984) was also mimicked by the application of IAA to roots. Application of increasing concentrations of IAA (10^{-9} to 10^{-4} M) to wheat seeds strongly decreased wheat root length, comparable to the effect seen upon inoculation with increasing concentrations of bacteria (Dobbelaere *et al.* 1999). Treatment with 10^{-6} M IAA can be compared to inoculation with 10^8 bacteria per mL, showing increased number and length of root hairs and a decreased root elongation zone as compared to control roots (Dobbelaere *et al.* 1999).

However, in some instances exogenous application of synthetic hormone did not simulate the effects induced by the bacterium in *Medicago polymorpha* seedlings (Yahalom *et al.* 1990), showing the controversy of the results. On the other hand, several investigations were conducted with mutant strains altered in IAA production to evaluate the involvement of bacterial IAA in the promotion of root development. *A. brasilense* SpM7918, a very low-IAA producer, showed a reduced ability to promote root system development in terms of both number and length of lateral roots and distribution of root hairs when compared to the wild type strain Sp6 (Barbieri and Galli 1993; Dobbelaere *et al.* 1999). Another mutant of *A. brasilense* with low production of phytohormones but high nitrogenase activity did not enhance root growth over uninoculated controls (Kundu *et al.* 1997). In short, several evidences support the involvement of IAA produced by *Azospirillum* in the promotion of plant growth. However, there are no reports showing to what extent IAA produced in the rhizosphere originates from *Azospirillum* (Steenhoudt and van der Leyden 2000).

The production of these regulators, in turn, could be influenced by compounds released by plant roots (Omay *et al.* 1993). As well as sugar released by roots is a C-source in feeding bacteria, it could also stimulate auxin synthesis in *Pseudomonas* spp. (Leinhos 1994). In this regard, the same root exudates that *in vitro* induce IAA production by *P. fluorescens* M.3.1 could be also stimulating in the rhizosphere the hormonal release near roots (Benizri *et al.* 1998). van de Broek *et al.* (1999) demonstrated that the expression of one of the main genes involved in two of the IAA biosynthetic pathways in *Azospirillum*, the *ipdC* gene: indole-3-pyruvate decarboxilase gene, is up regulated by auxins. In this sense, the presence of plant-derived auxins in the rhizosphere could be sufficient for *A. brasilense* to enhance the expression of the *ipd*C gene (Lambrecht *et al.* 2000).

Anyway, the possibility that *Azospirillum* could not only produce IAA but also enhance the endogenous IAA produced by the plant should not be excluded. Most studies on mechanisms for plant growth promotion by PGPR have focused on bacterial traits without examining the host plant's physiological responses (Bloemberg and Lugtenberg 2001). Moreover, the role of chemical signals in mediating belowground interactions is only beginning to be understood (Bais *et al.* 2006).

Bloom *et al.* (2003) have reviewed the signals and molecules that are potentially involved in root development. Among them, nitrogen species as ammonium, nitrate and NO are clearly implicated in root growth and proliferation. In this regard, it has been already demonstrated that NO functions as a signal molecule in the IAA-induced signaling cascade leading to ARD (Pagnussat *et al.* 2002, 2003). More recently, it was also reported that NO plays a central role during lateral root formation (LRF) (Correa-Aragunde *et al.* 2004), and root hair development (Lombardo *et al.* 2006).

It has been largely known that *Azospirillum* can produce NO at low O_2 pressure by denitrification (Hartmann and Zimmer 1994). The remarkable 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 ARD, LRF and root hair initiation and elongation, led us to explore whether the *Azospirillum* ability to promote root growth and change its architecture relies on NO. Recently, Creus *et al.* (2005) reported the NO production by this microorganism growing under aerobic conditions. A concentration of 6.4 nmoles of NO per gram of *A. brasilense* was quantified when the bacterium reached the end of growing log phase. In addition, *Azospirillum*-inoculated tomato roots incubated with a NO-specific fluorescent probe displayed higher fluorescence intensity compared to non-inoculated roots. Fluorescence was mainly located at the vascular tissues and subepidermal cells of roots (Creus *et al.* 2005). Moreover, the *Azospirillum*-mediated induction of LRF appears to be NO-dependent since treatment of inoculated seedlings with the NO scavenger-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide completely blocked this effect (Creus *et al.* 2005).

CONCLUSION

Plant-growth promoting microorganisms in vegetable crop production have received little attention by the scientific community. However, several evidences show that PGPMs could improve plant productivity in a human-deteriorated environment. In general, they could contribute to reduce the burden of soil nutrient loss in arable lands, to counteract part of the negative effects of water and saline stresses on plant growth, and to help plants avoid or minimize contaminants uptake. These results encourage new investigations on their application in horticulture. Moreover, common practices in vegetable production are soil solarization, transplants, explant seedings, drip irrigation with fertilization (fertigation), and use of non-soil supports. All of these could be improved by the use of PGPM. In addition, seed ageing and salt effects on lettuce germination could be ameliorated by *Azospirillum* inoculation. New evidence shed more light on the mechanisms of plant growth promotion exerted by this bacterium.

ACKNOWLEDGEMENTS

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