

Plant Beneficial Microbes and Their Application in Plant Biotechnology

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1. Introduction

Plants are involved in a complex network of interactions with microorganisms; some of those are beneficial, others are detrimental, but the former are by far the largest and still widely unexplored part. This chapter reviews the status of development and application of beneficial microbes that provide an option for future prospects.

There is a growing worldwide demand for sound and ecologically compatible environmentally friendly techniques in agriculture, capable of providing adequate nourishment for the increasing human population and of improving the quality and quantity of certain agricultural products. For these reasons, the application of beneficial microorganisms is an important alternative to some of the traditional agricultural techniques which, as it has been well documented, very often severely alter the agro-ecosystem balance and cause serious damage to health. For example, contamination of groundwater by leaching of nitrogen fertilizers, accumulation of nitrates and persistence of chemicals used in crop protection in edible portion of foods are cause of grave concern.

The use of beneficial microorganisms in the replacement or the reduction of chemicals has been so far attested (Dobbelaere et al., 2003; Burdman et al., 2000). Beneficial microorganisms such as diazotrophs bacteria, biological control agents (BCAs), plant growth promoting rhizobacteria (PGPRs) and fungi (PGPFs), can play a key role in this major challenge, as they fulfil important ecosystem functions for plants and soil (Whipps, 1997; Raaijmakers et al., 2009; Hermosa et al., 2011). Moreover, modern agriculture, based on the cultivation of a very limited number of crop species and cultivars, is susceptible to epidemic diseases traditionally contrasted through the use of chemicals. With most crops, no effective fungicides are available against a lot of fungal diseases. Plant growth stimulation and crop protection may be improved by the direct application of a number of microorganisms known to act as bio-fertilizers and/or bio-protectors. How beneficial

microorganisms really do act to improve plant rooting is only partially known, as several aspects have to be considered, including (i) the production of metabolites related to root development growth and pathogen control (phytohormones, antimicrobials, antibiotics), and (ii) the difficulty to discriminate the direct effects on the specific/total activities and the indirect effects due to the enhanced availability of nutrients and growth regulators.

Though over the past 150 years bacteria and fungi have been repeatedly demonstrated to promote plant growth and suppress plant pathogens, this knowledge has yet to be extensively exploited in agricultural biotechnology (Berg, 2009).

2. Plant-microorganism interactions: Ecological implications

Soil-borne microorganisms interact with plant roots and soil constituents at the root-soil interface, where root exudates and decaying plant material provide sources of carbon compounds for the heterotrophic biota (Barea et al., 2005; Bisseling et al., 2009). The number of bacteria in the rhizosphere (the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms) and rhizoplane (the external surface of roots together with closely adhering soil particles and debris) is higher than in the soil devoid of plants; this happens because soils devoid of plants are poor in many attractive substances secreted from the roots. As soon as a seed starts to germinate, a relatively large amount of carbon and nitrogen compounds i.e., sugars, organic acid, aminoacids, and vitamins are excreted into the surrounding environment. This attracts a large population of microorganisms inducing vigorous competition between the different species (Okon, 1994). Moreover, rhizosphere microbiomes typically differ between plant species (Bisseling et al., 2009).

Beneficial microorganisms are known to be biocontrol agents and/or growth promoters. There are several modes of action by which they can be beneficial to plant health, which can be related to an indirect or a direct positive effect. Microorganisms have indirect positive effects on plants, affecting adversely the population density, dynamics and metabolic activities of soil-borne pathogens, mainly through competition, antibiosis, lysis, and hyperparasitism. Competition takes place for space and nutrients at the root surface; competitive colonization of the rhizosphere and successful establishment in the root zone is a prerequisite for effective biocontrol. Antagonistic microorganisms can often produce a range of different antimicrobial secondary metabolites, and/or extracellular lytic enzymes. Hyperparasitism is well documented for *Trichoderma*; it involves secretion of chitinases and cellulases, contact with the pathogen, coiling of hyphae around the hyphae of the pathogen, enzymatic digestion of its cell wall, and penetration. Direct positive effects on plants are exerted by rhizosphere microorganisms through a phytostimulation and a biofertilization of plants; these processes involve production of phytohormones, non-symbiotic nitrogen fixation, and the increase of availability of phosphate and other nutrients in the soil (Burdman et al., 2000). Numerous compounds that are toxic to pathogens, such as HCN, phenazines, pyrrolnitrin, and pyoluteorin as well as, enzymes, antibiotics, metabolites and phytohormones are the means by which PGPRs act; similarly other phenomena such as quorum sensing and chemotaxis, are vital for rhizosphere colonization (Castro-Sowinski et al., 2007; Ramette et al., 2011; Jousset et al., 2011).

Under iron-limiting conditions of soil habitats and plant surfaces, PGPRs can produce low-molecular weight compounds called siderophores, that sequester iron in a competitive way,

thus depriving pathogenic fungi of this essential and often scarcely bioavailable element (Pedraza et al., 2007).

Many rhizosphere microorganisms can induce a systemic response in plants, activating plant defence mechanisms. Inoculation with non-pathogenic root zone bacteria can trigger signalling pathways that lead to higher pathogen resistance of the host, the so-called induced systemic resistance (ISR). Several of the bacteria that have been used to study beneficial effects under abiotic stress conditions, such as *Bacillus* sp., have been shown to induce ISR (Chakraborty et al., 2006). Some PGPRs elicit physical or chemical changes related to plant defense, a process often referred to as ISR, and/or tolerance to abiotic stress, such as drought, salt and nutrient excess or deficiency. For the latter PGPR-induced changes in plants, it has been proposed the term "induced systemic tolerance" (IST). IST relates to an enhanced tolerance to abiotic stresses (Yang et al., 2009). The metabolic pathways for signal transduction in plant defense responses can intercommunicate with other plant stress responses. In addition, the genes that are involved in plant responses to biotic and abiotic stresses can be co-regulated (Dimkpa et al., 2009).

The effect of the growth promotion exerted by PGPRs is mainly related to the release of metabolites and nitrogen fixation processes, the provision of bioavailable phosphorus for plant uptake, sequestration of iron by siderophores, production of plant hormones like auxins, cytokinins and gibberellins, and lowering of plant ethylene levels (Glick, 1995; Glick et al., 1999; Tortora et al., 2011). On the contrary, biocontrol occurs through an indirect action of the BCAs that interact with soil pathogens through several mechanisms such as antibiosis (production of antimicrobial compounds), competition for iron and nutrients or for colonization sites, predation and parasitism, induction of resistance factors (for example the plant is strongly stimulated to synthesize substance called phytoalexins, small molecules with antibiotic activity, which can inhibit the growth of many pathogenic microorganisms), production of enzymes such as chitinase, glucanase, protease and lipase (Whipps, 2001). Growth promotion and biocontrol can be due to the same microorganism that positively influences the development of the plant through different mechanisms, for instance the increased availability and assimilation of the mineral nutritional components, the release of growth factors and the suppression of pathogenic microorganisms. This is translated in more resistant and healthy plants. In addition, PGPR species are able to metabolize numerous and varying carbon sources, to multiply quickly and above all to show a greater competence in colonizing the rhizosphere in comparison to deleterious microorganisms.

The beneficial bacteria are widely studied by microbiologists and agronomists because of their potential in increasing plant production (Somers et al., 2004). The research involving the use of PGPRs were made mainly on herbaceous plants in open field environments and in horticultural crops. Moreover, their application has recently expanded both in forestry and in phytoremediation of contaminated soils. Strains belonging to the genera *Azospirillum* (Okon & Labandera-Gonzalez, 1994; Okon & Itzigshon, 1995; Dobbelaere et al., 2001), *Bacillus* (Reddy & Rahe, 1989; Kokalis-Bourelle et al., 2002; Kokalis-Bourelle et al., 2006) and *Pseudomonas* (McCullough et al., 1996; Meyer et al., 2010) have been used in experimental tests on a wide range of economically important crops.

Endophytic bacteria, those bacteria that dwell intercellularly in association with plants for most, if not all, of their life cycles (Bacon & Hinton, 2007), have been used for biological

control of various plant diseases, as well as for enhanced plant agronomic characteristics, such as increased drought tolerance and nitrogen efficiency.

These bacteria, that include anaerobic, aerobic, and microaerobic species, live within the intercellular spaces of plant, where they feed on apoplastic nutrients, as non-pathogens. They can be found within a wide variety of plant tissue, including seeds, fruit, stems, roots and tubers (Surette et al., 2003). Among them are comprised bacterial diazotrophs that do not form nodules on hosts, such as *Azospirillum* species, and some *Rhizobium* species. Isolated from a large diversity of plants (Rosenblueth & Martínez-Romero, 2006), in general they occur at lower population density than rhizospheric bacteria or bacterial pathogens and can positively affect host plant growth (Long et al., 2008). What makes bacterial endophytes suitable as biocontrol agents is their colonization of an ecological niche similar to that of phytopathogens (Ryan et al., 2008).

Endophytes can be strictly dependent on the host plant for their growth and survival ("obligate endophytes"); alternatively, "facultative endophytes" have a stage in their life cycle in which they exist outside host plants (Hardoim et al., 2008). The latter group probably comprises the vast majority of the microorganisms that can thrive inside plants. These endophytes often originate from the soil, initially infecting the host plant by colonizing, for instance, the cracks formed in lateral root junctions and then quickly spreading to the intercellular spaces in the root. Hence, to be ecologically successful, endophytes that infect plants from soil must be competent root colonizers. Endophytic colonization of the plant interior is presumably similar, at least in the initial phases, to colonization of plant roots by rhizobacteria. Competitive rhizosphere bacteria, for example members of the genera *Pseudomonas* (e.g. *P. fluorescens*), *Azospirillum* (e.g. *A. brasilense*) and *Bacillus* (Pedraza et al., 2007; Mano & Morisaki, 2008), are often also found as colonizers of the internal tissue of plants. A suite of environmental and genetic factors is presumed to have a role in enabling a specific bacterium to become endophytic. Inside the plant tissues, modulation of plant physiology by tinkering with the plant ethylene levels has emerged as a major strategy, because any effect on this plant stress signal has major impacts on the bacterial niche. How bacteria modulate plant ethylene concentrations is the key to their ecological success or competence as endophytes. The concept of "competent endophytes" has been proposed as a way to characterize those bacteria that possess key genetic machinery required to colonize the endosphere and to persist in it. This is in contrast to "opportunistic endophytes", which are competent rhizosphere colonizers that might become endophytic by coincidentally entering root tissue, but lack genes that are a key to their ecological success inside the plant. Moreover, it is possible to distinguish "passenger endophytes" that, in the absence of any machinery for efficient root colonization or entry, might enter plants purely as a result of chance events (Rosenblueth & Martínez-Romero, 2006; Mercado-Blanco & Bakker, 2007).

Bacterial endophytes, used for biological control of various plant diseases and for improved plant agronomic characteristics, may be of particular interest as they have the advantage of being relatively protected from the competitive soil environment; moreover, they usually grow in the same plant tissue where bacterial plant pathogens are detected (Bulgari et al., 2009). Their importance to crop production systems is only just beginning to be appreciated: so far, they have been shown to promote growth in potatoes, tomatoes, and rice, and they have been shown to be capable of inducing both biotic and abiotic stress resistance (Surette et al., 2003). A large number of mechanisms are being proposed to explain this effect:

production of antimicrobial compounds, macronutrient competition, siderophore production, induced systemic resistance. This array of proposed mechanisms reflects the high diversity of endophytic bacteria.

2.1 Tolerance to salinity

Soil salinity in arid regions is frequently an important limiting factor for cultivating agricultural crops. PGPR-elicited plant tolerance against salt stress has been intensively studied, showing that inoculation with endophytic bacteria can mitigate the effects of salt stress in different plant species.

High K^+/Na^+ ratios were found in salt-stressed maize in which selectivity for Na^+ , K^+ and Ca^{2+} was altered upon inoculation with *Azospirillum* (Hamdia et al., 2004).

Similarly, inoculation of pepper with *Bacillus* sp. TW4 led to relief from osmotic stress, which is often manifested as salinity (and/or drought) stress. In these plants, genes linked with ethylene metabolism under abiotic stress were down-regulated (Sziderics et al., 2007). Because *Bacillus* sp. TW4 showed ACC deaminase activity, the authors speculated that the enzyme may be involved in the lower expression of these genes. Salt stress has also been shown to affect nodulation during *Phaseolus-Rhizobium* interaction. However, secondary inoculation of the salt-stressed plants with *Azospirillum* caused an extended exudation of plant flavonoids compared to *Rhizobium* alone, implying an induction of flavonoid genes in the presence of *Azospirillum* (Dardanelli et al., 2008). Thus, the co-inoculation of plants with different bacterial species may contribute to relieving abiotic stress.

IST to salt stress was also noted with *Arabidopsis* (Zhang et al., 2008) using *Bacillus subtilis* GB03, a species that has previously been used as a commercial biological control agent. Interestingly, some of the volatile organic compounds (VOCs) that are emitted from *B. subtilis* GB03 (Ryu et al., 2004) are bacterial determinants involved in IST. The response to saline stress has also been evidenced in barley seedlings where inoculation with *Azospirillum* seemed to mitigate NaCl stress (Zawoznik et al., 2011).

2.2 Drought tolerance

Land surface becoming arid or semi-arid has been rising progressively in these last decades; water use efficiency is a current priority for the United Nations policy and a key issue for plant research. Under water stress conditions, leaf transpiration and leaf conductance decrease, and the water use efficiency rises; this mechanism keeps plant growth under water-limited environments (Aroca & Ruiz-Lozano, 2009).

Plant responses to drought include an increase in abscisic acid (ABA) levels, that cause stomatal closure to minimize water loss; these events involve production of activated oxygen species (Cho et al., 2008). Other plant-signalling compounds are involved in regulating stomatal closure, such as methyl jasmonate, salicylic acid and ethylene.

The mechanisms that allow plants to cope with drought stress are regulated by changes in gene expression; drought regulated genes can be divided in two groups: functional genes (encoding for transporters, detoxification enzymes, osmolyte biosynthesis enzymes etc.) and regulatory genes, that encode for transcription factors (Aroca & Ruiz-Lozano, 2009). On the

whole, the beneficial effects of PGPR on plant drought tolerance is caused by changes in hormonal contents, mainly that of ABA, ethylene and cytokinins.

ABA is involved in the enhancement of plant drought tolerance by PGPR; *Arabidopsis* plants inoculated with *A. brasilense* Sp245 showed more ABA content than non-inoculated ones (Cohen et al., 2008).

Different strains of *A. lipoferum* were used to inoculate wheat seedlings under drought stress. Inoculation alleviated the plant drought stress, increasing wheat growth and yield; different strains performed differently (Arzanesh et al., 2011).

Exactly how a beneficial bacterium induces changes in plant root morphology is not yet clear. Bacterial production of hormone-like substances and their ability to stimulate endogenous hormone levels were believed to play the key role in this process (Dobbelaere et al., 1999). However, more recently, it has been found that, under aerobic conditions, *A. brasilense* produces significant amounts of the small diffusible gas, nitric oxide, which has been shown to act as a signalling molecule in an IAA-induced pathway involved in adventitious root development (Creus et al., 2005; Molina-Favero et al., 2008).

At the transcriptional level, the bacterium *P. polymyxa* caused the induction of a drought-responsive gene, *ERD15*, isolated from drought-stressed *A. thaliana* (Timmusk & Wagner, 1999). The inoculated plants were more tolerant to drought stress than non-inoculated ones; that could be caused by a mild biotic stress that could help plants cope with subsequent drought stress.

3. Experimental considerations about plant-beneficial bacteria

In the Mediterranean area the use of microorganisms became indeed widespread in the '80s, in coincidence with the sudden spread of soybean crop, that required the inoculation of the nitrogen fixing *Bradyrhizobium japonicum*, mainly applied to seeds as peat based or liquid inocula at sowing. Operators became familiar with the use microorganisms in agriculture. After that, despite the huge potentiality of beneficial microorganisms, a relative low diffusion must be highlighted, owing to "inconsistent" results in field experiments, but also owing to prejudices derived from the easy and large availability of chemicals. At the moment, as a consequence of (i) a growing interest towards low input agriculture systems (organic farming, biodinamics, natural farming), (ii) a favourable opinion of consumers for food with no chemicals, and (iii) the increased difficulties in the employment of chemicals according to the most recent laws, we are assisting at a "microbiological revolution", and the use of microorganisms is increasing.

The use of beneficial microorganisms is mostly oriented to improve plant growth and protection in an agricultural context, nevertheless several applications in a wider environmental sense could be prospected, as reported by our group in scientific literature. *Pseudomonas fluorescent* (Russo et al., 1996; 2001; 2005), *Bacillus subtilis* (Felici et al., 2008), *Rhizobium* spp (Toffanin et al., 2000; Casella et al., 2006), are some of beneficial bacteria applied in our experimental/scientific work as biofertilizers and/or biocontrol agents in agriculture. Other potential applications currently include micropropagation, bioremediation and phytoremediation, phosphate solubilization, soil aggregation, sewage treatment, bioleaching, oil recovery, coal scrubbing and biogas production.

Azospirillum brasilense is a free-living, aerobic Gram-negative bacterium, that fixes N₂ under microaerobic conditions, highly motile, displaying a mixed pattern of flagellation, which offers these microorganisms the advantage of moving towards favorable nutrient conditions. These bacteria have been isolated in particular from the rizosphere of cereals and grasses, in soils with low organic content and low doses of nitrogen fertilization (Dobbelaere et al., 2001; 2003). They are able to penetrate the roots and grow endophytically in the intercellular spaces; they have been isolated from a large variety of soils and locations worldwide, in tropical and temperate regions (Steenhoudt & Vanderleyden, 2000). The *Azospirillum* species are plant growth promoting rhizobacteria, which positively affect the growth and the yield of many plants of agricultural and ecological importance (Bashan et al., 2004). Since the '80s, *Azospirillum* species have been extensively studied for their potential in improving the growth and yield of cereal crops, particularly in sub-tropical regions, firstly speculating that their ability in freely fixing nitrogen could improve soil fertility and increase nutrient uptake of plants. In the last years, much evidence has arisen that the beneficial effects of *Azospirillum* species depend on an array of contributions, such as production of phytohormones and other bioactive substances, rather than the bacterial nitrogen fixation. Multiple mechanisms are currently suggested to explain the beneficial effects on plant growth (Bashan & de-Bashan, 2010). *Azospirillum* is used in many Countries as bacterial inoculant, alone or together with other bacteria and vesicular arbuscular mycorrhizal (VAM) fungi, for many crops (Bashan et al., 2004). The effects on the yield have not been reported to be always positive; they depend on the bacterial strain, the inoculated plant cultivar, and the environmental conditions (Pandey et al., 1998). In particular, temperature has turned out to be of crucial importance when this bacterium is inoculated in winter crops, where responses can be low or non-significant (Kaushik et al., 2001). Hence, the growth response of inoculated crops is not completely predictable (Hartmann & Bashan, 2009); nevertheless, much progress has been made in this field, and the practical field application of *Azospirillum* is expanding worldwide, especially in Central and South America. It has been estimated that there were 300,000 ha inoculated fields in Mexico in 2007, while in Argentina over 220,000 ha of wheat and corn were inoculated in 2008.

Azospirillum brasilense has been proposed in our studies in different fields, ranging from agriculture (crops, micropropagation, grape and olive propagation, ornamental plants nursery) to environmental sciences (bioremediation, environmental engineering), as reported below.

3.1 Micropropagation

Micropropagation is an efficient method of propagating large numbers of genetically uniform plants (Honda & Kobayashi, 2004), although serious problems concerning specific steps including explant sterilization, media manipulation, and acclimatization phase, can often invalidate its success, making the plantlets production a cost-intensive process.

In vitro bacterization of potato plantlets has been shown to enhance their transplant stress tolerance thereby eliminating the need of an expensive greenhouse hardening step, which even now is commonly used by pre-elite seed potato producers. Plants bacterized *in vitro* with *Pseudomonas fluorescens* strains CHA0 and IP10 were found to have a significantly higher fresh shoot weight compared to non-bacterized plants in the same system. Arbuscular mycorrhizal (AM) fungi have also been shown to reduce drought stress and increase disease resistance.

We have investigated the possibility of using the PGPR *Azospirillum brasilense* Sp245 to improve the micropropagation of cherry plum (*Prunus cerasifera*) trees (Russo et al., 2008). We have examined the ability of *A. brasilense* to promote rooting of explants during *in vitro* culture in growth-chamber tests and to promote plant growth and plant health during *ex vitro* acclimatization in greenhouse. In the presence of indolebutyric acid (IBA), both rooting and growth of *P. cerasifera* cuttings were significantly improved by bacteria inoculation. During the acclimatization phase, the main positive effect of inoculation was an increased biomass production, as compared with uninoculated control, suggesting that during acclimatization the rhizobacterium produced phytohormones, increased the nutrient uptake of the roots, and caused an improvement of overall plant performance. An ability to protect plants against pathogen attack was also observed, with a plant survival rate of nearly 100% in inoculated plants as compared to 0% in the negative control. This protective effect was demonstrated both *in vitro* and *in vivo* against the pathogenic fungus *Rhizoctonia* spp.

The effectiveness of *A. brasilense* cells application to micropropagated plantlets at the time of transplanting from *in vitro* culture to acclimatization conditions, has been furthermore assessed on three different fruit tree rootstocks: Mr.S2/5 (*Prunus cerasifera* X *P. spinosa*), GF 677 (*Prunus persica* X *P. amygdalus*), MM 106 (Northern spy X M1). This is a critical phase, in which plantlets are subjected to numerous environmental stresses that may lead to significant plant loss. Plant growth and plant health at the end of *post-vitrum* acclimatization, both in growth-chamber and greenhouse trials, respectively, were evaluated.

After 60 days, growth parameters were positively affected by Sp245 inoculum. In the case of Mr.S 2/5, an increase in rootstock stem length and node number by 37% and 42%, respectively, compared to the control was noted. In the case of GF 677, the bacterial inoculum increased stem length and node number by up to the 75% and 65%, respectively, compared to the control. The inoculum did not exert on MM 106 for both parameters suggesting that the effects of Sp245 could depend on a specific clone-microbe association. In all the cases, however, a higher vigor, consistent with a wider leaf area, was present in the inoculated plantlets demonstrating that the use of *Azospirillum* can significantly contribute to optimize plant performance during the phase of adaptation of plants to *post-vitrum* conditions (Vettori et al., 2010).

Considering that the main obstacles to an intensive and widespread use of beneficial microorganisms, at the commercial level, have been so far the so called "inconsistent" results in field application, mainly related to biotic and abiotic adverse environmental factors, the *in vitro* and *post vitro* inoculation with bacteria may be a way of overcoming a part of these difficulties.

3.2 Co-inoculation strategies

In recent years, a number of studies on co-inoculation of two or more beneficial microorganisms for better crop productivity have been reported. Positive effects, such as increase in biomass parameters, nitrogen-content and yield have been found in legumes inoculated with *Rhizobium* and *Azospirillum*. These positive effects may be attributed to early and increased nodulation, enhanced N₂ fixation rates, and a general improvement of root development. Stimulation of nodulation following the inoculation with *Azospirillum* may be derived from an increase in production of lateral roots, root hair density and branching, but

also from the differentiation of a greater number of epidermal cells into root hairs susceptible for infection by rhizobia. Nodulation by rhizobia co-inoculated with *Azospirillum* may also be enhanced by an increased secretion of root flavonoid substances that are involved in the activation of the nodulation genes in *Rhizobium* (Dobbelaere et al., 2001). Considerable results have also been obtained on grain yield, N, P, K content in wheat co-inoculated with *Azospirillum brasilense* and *Rhizobium meliloti* (Askary et al., 2009).

The effects of co-inoculation of bacteria and fungi has also been reported. *Azospirillum*-AM fungus combination seems suitable for sustainable agriculture practices, since both types of microorganisms are compatible with each other. The stimulatory effect of the *Azospirillum* inocula on root growth did not significantly influence the mycorrhization, regardless of the AM fungus involved, either in wheat or in maize plants, grown in the greenhouse and/or under field conditions. The effect of *Azospirillum brasilense* Sp245 was assessed at greenhouse level in three different cultivars of durum wheat, in the presence of indigenous AM fungi, and in maize plants artificially inoculated with *Glomus mosseae* and *Glomus macrocarpum*. At field level, the establishment of natural AM fungal symbiosis was evaluated with the commercial strain *Azospirillum lipoferum* CRT1 in maize plants (Russo et al., 2005). Positive effects of *Azospirillum brasilense* and arbuscular mycorrhizal colonization on rice growth and drought resistance have also been attested (Ruíz-Sánchez et al., 2011).

On the other hand, the combination of two rhizobacteria had no synergistic or comparable effects on plant biomass, with respect to their single applications. Indeed, individual inoculation of *B. subtilis* and *A. brasilense* Sp245 positively affected the growth in dry weight of both shoots and roots of tomato plants, but the combination of the two rhizobacteria had no synergistic or comparable effects on plant biomass. *In vitro* tests and cellular analysis of root tips revealed a growth inhibition of the primary root, which is not related to a reduced persistence in the rhizosphere of one or both bacteria (Felici et al., 2008). Moreover co-inoculation with mycorrhiza and rhizobia of different bean genotypes resulted in the reduction in the trehalose content and the authors concluded supporting the idea of using rhizobial or mycorrhizal inoculation separately (Ballesteros-Almanza et al., 2010).

These results suggest that mixing different microorganisms in the same inocula/treatment can cause interferences and consequent bad or lower than expected performances. Interactions and antagonist phenomena in contaminant species against *Bradyrhizobium japonicum*, obtained from the same soybean inocula preparation, had already been evidenced in the '80s.

3.3 Bioremediation and phytoremediation

Phytoextraction, actuated by hyperaccumulating or non-hyperaccumulating species, could be improved by using a plant-microbe system (Zhuang et al. 2007), thus contributing to novel promising methods for the cleaning-up of soils contaminated by heavy metals. Rhizobacteria of the genus *Azospirillum* have been extensively used for crop phytostimulation as above stated, thanks to the positive interaction between bacteria and plants at root level (Dobbeleare et al., 2001; Dobbeleare et al., 2003; Russo et al., 2005; Russo et al., 2008).

The implementation of lead phytoextraction in contaminated industrial soils by applying *A. brasilense* Sp245 to plants of indigenous species belonging to Mediterranean forestry was investigated. The possible phytoextraction ability was evaluated in *Myrtus communis* L. and *Laurus nobilis* L., previously selected among other plant species that were found able to grow in the contaminated areas, on the basis of the Pb content (Emission

Spectrophotometer Atomic Plasma, ICP-AES), the growing speed and the vegetative *habitus*. By trials carried out in greenhouse, it was shown that *A. brasilense* Sp245 can enhance the plant growth in Pb contaminated soil and affect the plant total lead content. Greenhouse trials were performed for 2 and 9 months, and plants were grown in pot in the presence of two level of Pb (312 and 4345 ppm).

The presence of Sp245 positively affected the total amount of Pb that was removed by plants, either as total biomass produced (Figure 1) or as specific Pb concentration, as a consequence of the incremented root growth, attesting the synergic effect of plants and microorganisms in a bioremediation system, and as higher specific Pb concentration (Table 1 and Table 2). Moreover the bioconcentration factor (Pb in plant tissues/Pb in soil) and translocation factor (Pb in leaves and shoots/Pb in roots) were significantly affected by the presence of *A. brasilense* Sp245, attesting the synergetic effect of plants and microorganisms in a rhizoremediation system.

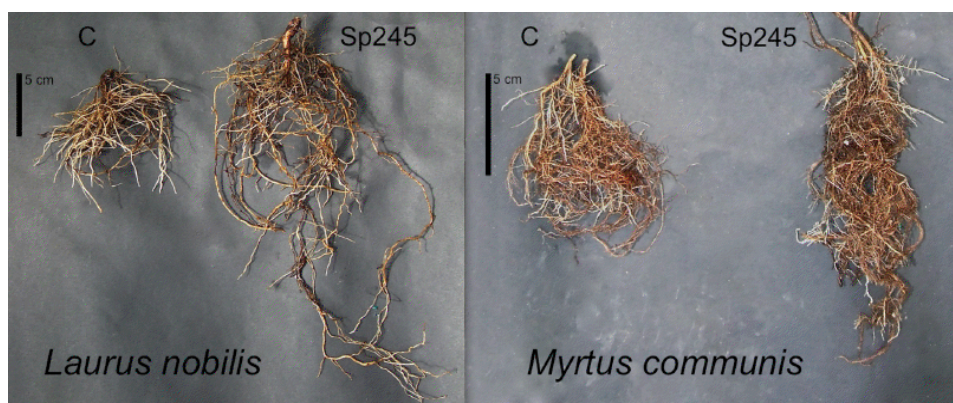


Fig. 1. Effect of *Azospirillum brasilense* Sp245 inoculation on root system in *Laurus nobilis* and *Myrtus communis* after nine months of pot cultivation with Pb polluted soil.

Shrub species	Pb uptake (mg/Kg dry matter)		Pb uptake Effect of <i>A. brasilense</i> Sp 245	
	Pb-	Pb+	Pb-	Pb+
<i>Laurus nobilis</i>				
Two months	63b	430a	77b	438a
Nine months	79c	466b	94c	534a
<i>Myrtus communis</i>				
Two months	103c	806b	116c	954a
Nine months	191d	1176b	305c	1324a

Table 1. Effect of *A. brasilense* Sp245 inoculation on Pb uptake (mg/Kg d.m.) by each plant of *Laurus nobilis* and *Myrtus communis* after two and nine months of pot cultivation with soils at different Pb concentration (Pb- : 312 mg/Kg and Pb+ : 4345 mg/Kg). Within each shrub species and each period means with the same letter were not significantly different according to the SNK'test ($P \leq 0.05$).

Shrub species	Control	Effect of <i>A. brasilense</i> Sp 245
<i>Laurus nobilis</i>		
Two months	3.1c	5.6b
Nine months	6.6a	7.5a
<i>Myrtus communis</i>		
Two months	2.9d	5.2b
Nine months	4.1c	6.3a

Table 2. Effect of *A. brasilense* Sp245 inoculation on biomass produced (g/plant d.m.) by *Laurus nobilis* and *Myrtus communis* after two and nine months of pot cultivation with Pb polluted soil. Within shrub species means with the same letter were not significantly different according to the SNK' test ($P \leq 0.05$).

3.4 Naturalistic engineering and endotherapy

The increased root density and branching, the improving in rooting of cuttings and the better adaptation to biotic and abiotic stresses derived from beneficial microorganisms, may represent an advantage in soil bioengineering and landscape construction. The role of vegetation in slope stability and restoration of steep rock faces with shrubs and trees is difficult due to extreme microclimatic and edaphic conditions (Beikircher et al., 2010). Inoculation with *A. brasilense* Sp245 of plant material used for restoration of drought-prone sites during preconditioning, can increase the drought tolerance and can play a synergetic and pivotal role in that phase. Work in progress with some angiosperm species, known for their vulnerability, gives us good expectation.

Another promising field could be the use of selected beneficial microorganisms in endotherapy, or trunk injection. This is an alternative method of treatment of urban woodland plant, with many advantages compared to traditional air treatments, including the absence of spraying of chemicals, and hence the complete harmlessness for the health of citizens, birds and other animals (Sánchez-Zamora & Fernández-Escobar, 2004; Hubbard & Potter, 2006).

4. Conclusions

Agriculture is the oldest economic sector in the world, and is more dependent on fertile soils and a stable climate than any other trade. At the same time, it has a huge influence on the ecological balance, water and soil quality, and on the preservation of biological diversity. Since the middle of the last century, agricultural techniques and economic framework conditions worldwide have undergone such a radical transformation that agriculture has become a major source of environmental pollution.

The investigation about ecologically compatible techniques in agriculture and environmental sciences can take essential advantage from the use of beneficial microorganisms as plant-microbe interactions fulfil important ecosystem functions.

Plant diseases are a major cause of yield losses and ecosystem instability worldwide. Use of agrochemicals to protect crop against plant pathogens has been increasing along with the intensification of agricultural production over the last few decades.

New biotechnological methods for crop protection are based on the use of beneficial microorganisms applied as biofertilizers and/or biocontrol agents; this approach represents an important tool for plant disease control, and could lead to a substantial reduction of chemical fertilizer use, which is an important source of environmental pollution. Nevertheless, despite dedicated efforts to study beneficial microorganisms, relatively few products have been registered for agricultural use and they count for a very small fraction of the potential market. This is a consequence of several limitations affecting their commercial expansion, which are mainly related to the survival capability of microorganisms under various environmental conditions.

Moreover, nowadays, microbial inoculants, some of which have a historical record for safe use since 1896 (the well-known rhizobia, for the inoculation of legumes) or since the 1930s (e.g. *Bacillus thuringiensis*, for the biological control of invertebrate pests) are being widely applied in modern agriculture as biofertilizers and biocontrol agents. Other interesting applications include micropropagation, bioremediation and phytoremediation, phosphate solubilization, soil aggregation, sewage treatment, bioleaching, oil recovery, coal scrubbing and biogas production, and represent incoming fields of application.

In short, from the examples and references cited above, it is manifest that useful microorganisms of agricultural importance represent an alternative and ecological strategy for disease management, in order to reduce the use of chemicals in agriculture and to improve cultivar performance. At the same time, their application is a highly efficient way to resolve environmental problems, for example through bioremediation and bioengineering. However, although beneficial microorganisms hold a great promise for dealing with different environmental problems, it is important to acknowledge that much of this promise has yet to be realized. Indeed, much needs to be learned about how microorganisms interact with each other and with the environments. For the future development of biotechnology in this field, the contribution of a combination of scientific disciplines is of primary importance to promote sustainable practices in plant production system, as well as in conservation and ecosystem restoration.

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