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# Legume Crops, Importance and Use of Bacterial Inoculation to Increase Production

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## 1. Introduction

Legumes are flowering plants that produce seedpods. They have colonized several ecosystems (from rain forests and arctic/alpine regions to deserts; Schrire et al., 2005), and have been found in most of the archaeological record of plants. Early in 37 B.C. Varro said “Legumes should be planted in light soils, not so much for their own crop as for the good they do to subsequent crops” (Graham & Vance, 2003), recognizing the importance of multiple cropping and intercropping production.

Leguminosae or Fabaceae is the third most populous family of flowering plants (behind Asteraceae and Orchidaceae) with 670 to 750 genera and 18,000 to 19,000 species. Legumes include important grain, pasture and agro-forestry species. They are harvested as crops for human and animal consumption as well as used as pulp for paper production, fuel-woods, timber, oil production, sources of chemicals and medicines, and are also cultivated as ornamental, used as living fences and firebreaks among others (Lewis et al., 2005).

The legumes provide many benefits to the soil so they are usually utilized as cover crop, intercropped with cereals and other staple foods. They do produce substantial amounts of organic nitrogen (see below, Improving legume yield by inoculation with rhizobia), increase soil organic matter, improve soil porosity and structure, recycle nutrients, decrease soil pH, reduce soil compaction, diversify microorganisms and mitigate disease problems (U.S Department of Agriculture [USDA], 1998). In rotation with cereals, legumes provide a source of slow-release nitrogen that contributes to sustainable cropping systems. The improvement in the production of these crops will therefore contribute substantially to better human nutrition and soil health (Popelka et al., 2004).

Based on total harvested area and production, cereals are the most important crops, and they are followed by legumes (Fig.1). Close up to 180 million Ha (12-15% of the Earth's arable surface) are worldwide used to produce grain and forage legumes. These numbers point the central importance of world legumes production. In addition, the promise of low-cost production of legume biomass, mainly soybean, for bioenergy purpose focus attention of investors in the improvement of legume production, and deserves an entirely section for discussion.

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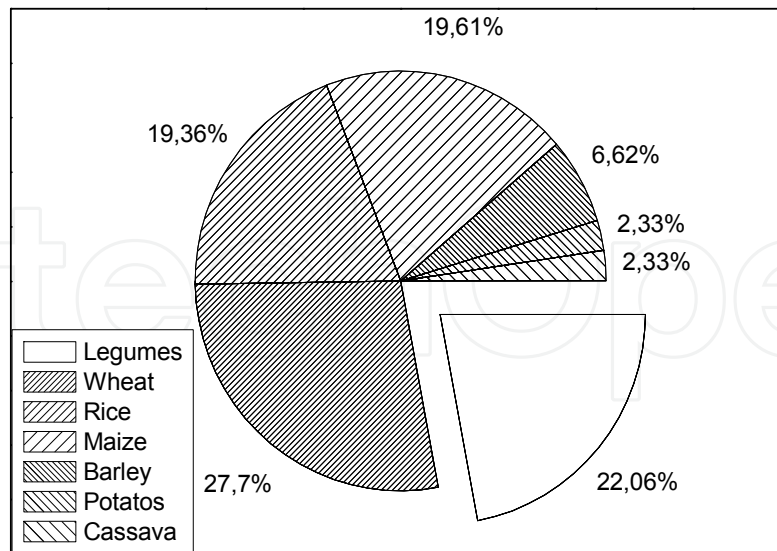


Fig. 1. Net worldwide harvested area for several crops, in the year 2009. Data obtained from Food and Agriculture Organization database [FAO] (<http://faostat.fao.org/site/567/default.aspx#ancor>) and Graham & Vance (2003).

### 1.1 Forage and grain legumes

Forage legumes play an important role in dairy and meat production being sources of protein, fibre and energy. They are usually richer in protein, calcium, and phosphorus than other non-legume forages, such as grass. They include alfalfa (*Medicago sativa*), clover (*Trifolium spp.*), birdsfoot trefoil (*Lotus corniculatus*) and vetch (*Vicia spp.*) among others. Alfalfa is one of the most important forage crops. In 2006, the worldwide production was around 436 million tons. U.S. is the largest alfalfa producer, with 15 million Ha planted in 2010. Canada, Argentina (primarily grazed), Southern Europe, Australia, South Africa, and the Middle East have also considerable production (FAO, 2011).

Grain legumes also called pulses, which according to FAO are crops harvested exclusively for the dry seeds, play an important role in the nutrition of many people due to their high protein content in seeds. They represent a major source of protein in many developing countries, especially among the poorest population, and are rich in essential amino acids such as lysine, supplementing thus the nutritional value of cereal and tuber diets (Graham & Vance, 2003). The world pulse production has almost increased by half during the period of 1980 - 2004, overtaking the 60 million tons in 2005 (FAO, 2005). According to FAO Statistical Yearbook 2010, in the year 2008, Canada, China and United States were the main exporters of pulses (28%, 12% and 11% of total exports, respectively). Interestingly, India, the world's 12<sup>th</sup> largest economy and the third largest in Asia behind Japan and China, is the main importer, responsible of 21% of global trade in of pulses (2.5-3.5 million tons). India produces (15-18 million tons; the world's largest producer), imports and consumes (18.5-20.5 million tons) a wide range of pulses. Thus, considering pulse relevance in the world's largest economies such as U.S., China and India, incomes and a raising world population, it is obvious the interest of farmers and investors for improving pulse production.

## 1.2 Soybean – The new legume-star

The soybean (U.S.) (*Glycine max*), also called soya bean (U.K.), is an annual summer legume native of South-eastern Asia, which is used as human food (Liu, 1999) and livestock feed as well as for several industrial purposes (Ali, 2010). According to the newest available information, this legume is one of the main crops cultivated for oil extraction (35.9 million tons oil and 57% global oilseed production), preceded only by the oil of palm (FAO, 2011). Interestingly, over half of the world's 2007 soybean crop (58.6%) was genetically modified (GM), achieving 77% in the year 2009. These GM-soybeans possess a gene that confers herbicide resistance. The nations that produce almost exclusively GM-soybean are U.S. (85%) and Argentina (98%), tending to 100%. The global production and utilization of soybean have increased by ten during the last century (Qiu & Chang, 2010). In 2009, world's soybean cultivated area and production were 99.5 million Ha and 223.2 million tons (FAO, 2011), respectively. U.S. is the world's leader soybean producer and exporter, responsible of 41% global production, followed by Brazil (26%), Argentina (14%), China (7%) and India (4%) (FAO, 2011).

In U.S. the soybean farm gate value raised more than double, ranging from 12.6 billion USD (in 2001) to 29.6 billion USD (in 2009). The price of soybean has increased more than 80% because of soybean-oil's use in soy-biodiesel and as feed for fish farming. Biodiesel is in demand and soybean represents about 25% total worldwide global biodiesel raw material (Pahl, 2008). The net energy balance when the soybean-oil is used for fuel has improved since soybean is a legume, it fixes nitrogen and does not require nitrogen fertilizer (see below) (Kinney & Clemente, 2010).

## 2. Improving legume yield by inoculation with rhizobia

Leguminous plants are relevant economic and cultural important crops because their exceptional diversity, manifested in variety of vegetable forms that adapted to a wide range of ecological conditions, the high protein content of some grains, their use as pastures, increased world production and commodities. In this scenario, many farm investors, industries and researchers have focussed attention in the development of biological and eco-friendly technologies for legume growth improvement and establishment. The ability of many legumes to form associations with bacteria that fix atmospheric nitrogen (the symbiotic association that improve growth) is thus a big matter of ecological and economic interest (Zahran, 2009).

### 2.1 Biological vs chemical nitrogen fertilization

Microorganisms are essential to the Earth's nitrogen cycle and to the Biological Nitrogen Fixation (BNF) process in leguminous plants, playing a very important role in terms of plant production in agriculture. Nitrogen fixing microorganisms could be used in live formulations (biofertilizer) that when applied to seed, root or soil colonize the rhizosphere, or the interior of the plant, and promote growth by increasing the nitrogen supply to the host plant and building up soil health. The evaluation, in terms of economic and ecological costs, between chemical- and biological-nitrogen fertilizers support that BNF represents an economic, sustainable and environmentally friendly resource to guarantee the nitrogen requirement of an agro-ecosystem.

Chemical-fertilizer demand has historically been influenced by changing and often interrelated factors such as increasing populations and economic growth, agricultural production, prices, and government policies. In 2007, the production of chemical nitrogen fertilizers was 130 million tons which is likely to increase further in the coming years (FAO, 2011). Their production requires a great consumption of fossil fuels (1-2 % global fossil fuel) and is subjected of constant variations in prices (Vieira et al., 2010). Although their direct contribution to energy consumption seems minimal, it is unnecessary and unsustainable. On average, U.S. farmers apply 30-40 % more chemical nitrogen than is needed for optimal crop yield, thus wasting most of the applied chemical nitrogen. Given the rising cost of chemical nitrogen fertilizers, nitrogen fixation cover crops offer significant economic benefits. In 2006, the price of nitrogen fertilizers in U.S. raised to 521 USD per ton (Huang, 2007), estimating an over cost of 7 to 10 billion USD annually compared with FBN. For instance, the modest use of alfalfa in rotation with corn by U.S. farmers saved 200 to 300 million USD (Graham & Vance, 2003).

In addition to the inconvenience of increasing prices, chemical nitrogen fertilization is associated with environmental problems because watershed contamination by nitrogen leaching, volatilization and denitrification. These problems could be avoided offering to farmers low-cost biofertilizer technologies. These are ecologically sound and their application could help to minimize the global warming as well as to reduce the fertilizer input in farming practices (Herridge et al., 2008a).

## 2.2 The biological nitrogen fixation (BNF)

BNF benefits not only the legumes themselves but also any intercropped or succeeding crop, reducing or removing the need for nitrogen fertilization. In soils with low mineral nitrogen content, nitrogen fixing microorganisms provide ammonium into the legume biomass, allowing faster growing than their plant competitors. In contrast, if nitrogen is abundant, nitrogen fixing microorganisms tend to be competitively excluded by non-fixing species because the nitrogen fixation process is bio-energetically costly (Houlton et al., 2008). It means that there is a range of physiological and ecological situations that tend to constrain BNF in legume systems, mainly by the nitrogen demand of the plant and by the C:N stoichiometry of the ecosystem. In fact, the hypothesis of a feedback control between legume demand and BNF in a particular ecosystem has been now supported by evidence from both experimental and theoretical models (Soussana & Tallec, 2010).

There is the potential to increase BNF by the use of well adapted and efficient nitrogen fixing microorganisms and/or genetic modified plant species to ensure legume crop at high levels of productivity. Farmers are familiar with the application of commercially available microorganisms (inoculants) that have been especially selected for their ability to effectively nodulate plants and to fix nitrogen from the atmosphere. These kind of microbial inoculants, also known as soil inoculants, are agricultural amendments that use microorganisms known as rhizobia to promote legume growth. These bacteria form symbiotic relationships with the target leguminous plant, and both parts benefit. The legume supplies energy and photosynthates to rhizobia, and rhizobia provide the legume with nitrogen, mainly in the form of ammonium (Howard & Rees, 1996). The symbiosis is initiated through the legume root infection by the rhizobia and formation of root nodules where BNF occurs through the action of a bacterial enzyme, called "Nitrogenase" (Masson-Boivin et al., 2009).



### 2.3 Rhizobia: The master microbe

The current taxonomy of rhizobia consists of several genera in the subclass Alpha- and Beta-Proteobacteria. *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Azorhizobium*, *Methylobacterium*, *Bradyrhizobium*, *Phyllobacterium*, *Devosia* and *Ochrobactrum* are genera that belong to rhizobial Alpha-Proteobacteria. In rhizobial Beta-Proteobacteria the following genera have been described: *Burkholderia*, *Herbaspirillum* and *Cupriavidus* (NZ Rhizobia, 2011). It is important to clarify that this classification is based on taxonomically important strains that may not necessarily be important reference strains for legume growth improvement. Rhizobial strains commonly used in inoculants have good field performance and stability of symbiotic properties in culture, but are not necessarily well documented or used in taxonomy or molecular biology studies (Lindström et al., 2010). The legume-rhizobia association is specific (each rhizobial strain establishes a symbiosis with only a limited set of host plants and *vice versa*). Thus, there is a restricted number of inoculants that fit with a leguminous plant, and farmers must know which inoculant must be applied according plants and characteristics of soil (Mabrouk & Belhadj, 2010). In other words "Be sure to buy the right inoculant for the legume the farmer intends to plant". Such information must be given by the manufacturer and should be clearly specified in the label. Plants mutually compatible with the same species of rhizobia were listed in earlier years in so-called "cross-inoculation groups" (Table 1). This concept was used in rhizobial taxonomy, but is it unreliable as taxonomic marker because of aberrant cross-infection among plant groups.

Rhizobia	Legume Cross-inoculation group
<i>Ensifer meliloti</i>	Alfalfa Group: alfalfa ( <i>Medicago sativa</i> ), sweet clover ( <i>Melilotus</i> spp.) (yellow and white), fenugreek ( <i>Trigonella</i> spp.)
<i>Rhizobium leguminosarum</i> bv <i>trifolii</i>	Clover Group (Clover I, II, III and IV): clovers ( <i>Trifolium</i> spp.)
<i>Bradyrhizobium japonicum</i>	Soybean Group: soybean ( <i>Glycine max</i> )
<i>Bradyrhizobium</i> spp.	Cowpea Group: pigeon pea ( <i>Cajanus cajan</i> ); peanut ( <i>Arachis hypogaea</i> ); cowpea, mungbean, black gram, rice bean ( <i>Vigna</i> spp.); lima bean ( <i>Phaseolus lunatus</i> ); <i>Acacia mearnsii</i> ; <i>A. mangium</i> ; <i>Albizia</i> spp.; <i>Enterlobium</i> spp., <i>Desmodium</i> spp., <i>Stylosanthes</i> spp., Kacang bogor ( <i>Voandzeia subterranea</i> ), <i>Centrosema</i> sp., winged bean ( <i>Psophocarpus tetragonolobus</i> ), hyacinth bean ( <i>Lablab purpureus</i> ), siratro ( <i>Macroptilium atropurpureum</i> ), guar bean ( <i>Cyamopsis tetragonoloba</i> ), calopo ( <i>Calopogonium mucunoides</i> ), puero ( <i>Pueraria phaseoloides</i> )
<i>Rhizobium leguminosarum</i> bv <i>viciae</i>	Pea Group: peas ( <i>Pisum</i> spp.), lentil ( <i>Lens culinaris</i> ), vetches ( <i>Vicia</i> spp.), faba bean ( <i>Vicia faba</i> )
<i>Rhizobium leguminosarum</i> bv <i>phaseoli</i>	Bean Group: beans ( <i>Phaseolus vulgaris</i> ), scarita runner bean ( <i>Phaseolus coccineus</i> )
<i>Mesorhizobium loti</i>	Chickpea Group: chickpea ( <i>Cicer</i> spp.), Birdsfoot trefoil ( <i>Lotus corniculatus</i> L.)
<i>Rhizobium lupini</i>	Group Lupines
<i>Rhizobium</i> spp	Crownvetch

Table 1. Cross-inoculation group and *Rhizobium*-legume association

The occurrence of a wide diversity of microorganisms in a particular soil increases the opportunity for a legume host to find compatible rhizobia. The principle of specific legume-rhizobia association is commonly used for the isolation of well adapted and efficient rhizobial strains (Castro-Sowinski et al., 2002; Florentino et al., 2010). Usually trap-plants are used to catch the rhizobial strain with highest performance and the strain is used for the design of new inoculants. Details about inoculation technology for legumes can be read in Herridge (2008b).

#### **2.4 Formulation and low-cost are crucial aspect of producing inoculants**

Formulation is the industrial “art” of converting a promising laboratory-proven microorganism into a commercial field product. But, the development of successful inoculants involves more than the selection of the most efficient rhizobial strain, it involves the choice of a carrier (powder, granule, and liquid), packaging and marketing, avoiding of microbial contaminations. Inoculant preparations for agricultural use constitute a stressful environment because bacterial cells may have to be stored for long periods, and should survive desiccation and transportation conditions. Some aspects related to inoculant preparation, production and application are described by Hungria et al. (2005).

The formulation should maintain or enhance activity in field. In order to survive in nutrient-poor ecosystems, bacteria use different strategies, among them, the use of polyhydroxyalkanoates (PHA) as intracellular carbon storage compounds. Cells with higher PHA content can survive longer than those with lower amounts, and PHA degraded elements can be used rapidly for numerous metabolic needs. Accumulation of PHA can provide the cell with the ability to endure a variety of harmful physical and chemical stresses (Castro-Sowinski et al., 2010; Kadouri et al., 2005).

A good formulation contains microorganisms (active ingredient) in an active metabolic state, immersed in a suitable carrier together with additives that are responsible for the microbial cells stabilization and protection during storage and transportation. Most of the research done in the improvement of inoculant quality is based on improving carrier properties, by adding elements that can prolong survival, such as nutrients, or other synthetic products (López et al., 1998). Most commercial inoculants are in powder (finely ground peat mixed with the nitrogen-fixing bacteria), ready for mixing with the seed. Granular formulations are designed to be placed in the seed rut at planting. Liquid inoculants and other non-peat-based inoculants are also being used. Liquid inoculants simplify the production of the inoculant and the application to seeds or field. However, bacterial survival in the inoculant and on inoculated seeds is not as good as when using peat as a carrier, because bacteria lack carrier protection (Tittabutr et al., 2007). Peat provides bacterial protection and prevents drying and death, compared to the inoculants that do not contain peat. However, alternative substrates to peat can be used as carriers: compost cork, perlite, volcanic pumice, alginate beads and coal, among many others, also gave good results in terms of supporting bacterial growth and long survival, as well survival on seeds (Albareda et al., 2008; Ben Rebah et al., 2007).

Another important consideration in formulation is the cost-effectiveness that must be low enough to allow sufficient incoming compared to chemical fertilization. In U.S. and Canada, a seed inoculant is sell for 5.00 and 2.50 USD per Ha, respectively, while granular inoculants

range from 15.00 to 18.00 (US) per Ha (Xavier et al., 2004). But, inoculants need only a modest increase in yield to offset the cost. A good inoculant will usually provide at least a 70- to 140-Kg per Ha return on yield.

### 2.5 The input of BNF in legume yield

The annual input of fixed nitrogen was calculated to be 2.95 Mton for the pulses and 18.5 Mton for the oilseed legumes, being the soybean the dominant crop legume (50% global crop legume area and 68% global production). In addition to the annual legume nitrogen fixation inputs of 12-15 Mton (pasture and fodder legumes), there is an input by nitrogen fixation in rice (5 Mton), sugar cane (0.5 Mton), non-legume crop lands (<4 Mton) and extensive savannas (<14 Mton). Thus, the total overall estimated in agricultural systems is of 50-70 Mton biologically fixed nitrogen (Herridge et al., 2008a). These numbers show that the process of BNF is an economically attractive and eco-friendly alternative to reduce the external nitrogen (chemical fertilizers) input, which improves the quality and quantity of crop resources.

A successful BNF is capable of improving agricultural productivity while minimizing soil loss and ameliorating adverse edaphic conditions. Conditions such as drought, salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, high temperature, insufficient or excessive soil moisture, inadequate photosynthesis, and plant diseases conspire against a successful symbiotic process. Many inoculant manufactures worldwide have developed formulations with high symbiotic efficiency under stress conditions. However, the actual view of plant growth promoting preparations focuses their investigations in the design and development of new-formulations supplemented with plant and/or microbe exudates. These exudates contain molecules involved in the microbe-plant interaction: flavonoids, sugars, acids, amino acids, amines and other low molecular weight compounds that promote plant growth (Skorupska et al., 2010; Garg & Geetanjali, 2009). Macchiavelli & Brelles-Mariño (2004) showed increased plant nodulation treating *Medicago truncatula* roots and seeds with Nod Factors prior to inoculation. Lipo-chito-oligosaccharides (LCOs), or Nod Factors (NFs), are bio-signals produced by the rhizobia which act as bacteria-to-plant communication molecule that mediates recognition and nodule organogenesis (Masson-Boivin et al., 2009). The inclusion of NFs in formulations might have technological applications since presoaking seeds with submicromolar concentrations of this oligo-saccharide before sowing led to increased nodulation under field conditions. In fact, a soybean inoculant based on NFs technology was introduced on the market many years ago (Zhang & Smith, 2002). Currently, many companies like Rizobacter ([www.rizobacter.com.ar](http://www.rizobacter.com.ar)) and Nitragin ([www.nitragin.com.ar](http://www.nitragin.com.ar)) are marketing formulations with bio-signals that improve the symbiotic relationship, activate mechanisms to resist abiotic stress conditions, and induce defensive response.

### 3. The use of microbial consortium in legume agronomic production

The new fashion in agriculture is the use of microbial consortiums of plant-growth promoting bacteria (PGPB, which includes rhizobia). PGPB are exogenous bacteria introduced into agricultural ecosystems that act positively upon plant development (Castro-Sowinski et al., 2007). It is possible to increase agricultural productivity and, eliminate or decrease the use of chemical fertilizers and pesticides (Adesemoye et al., 2009a; Vessey, 2003) even in marginal soils (Gamalero et al., 2009) when the formulation contains different PGPB.



### 3.1 Getting more from legumes

Current studies indicate that we are still detecting new bacteria and fungi with diverse growth-promoting characteristics, and that the combination of different PGPB into a single-formulation increases plant yield, compared with single-inoculation. On the other hand, efforts have been done manipulating PGPB to produce master inoculants by the introduction of foreign DNA that provides new abilities (GMM, Genetic Modified Microorganisms). Globally, it was expected a big explosion in this area of research, the use of recombinant DNA-technological tools for the production of inoculants (Barea et al., 2005; Valdenegro et al., 2001). However, the use of GMM is in discussion and needs clear regulatory policies, controls and suitable legislation (Fedoroff et al., 2010).

Some cooperative microbial activities can be exploited for developing new sustainable, environmentally-friendly, agro-technological practices (Barea et al., 2005). In this regard, the plant co-inoculation with rhizobia and other PGPB received considerable attention for legume growth promotion (Cassán et al., 2009; Bai et al., 2002a; 2002b; Zhang et al., 1996). Results from many studies concerning the effect of co-inoculation on legume growth are summarized in Table 2. Several genera of bacteria have been identified as “helpers” of the rhizobia-legume symbiotic process (Beattie, 2006). Examples are bacteria of the genus *Azospirillum* (Cassán et al., 2009; Itzigsohn et al., 1993), *Azotobacter* (Qureshi et al., 2009; Yasari et al., 2008), *Bacillus* (Bullied et al., 2002), *Pseudomonas* (Barea et al., 2005; Fox et al., 2011), *Serratia* (Bai et al., 2002b; Lucas-Garcia et al., 2004a; Zhang et al., 1996), *Thiobacillus* (Anandham et al., 2007), and *Delftia* (Morel et al., 2011), among many other. The stimulation of the legume-rhizobia symbiosis by non-rhizobial-PGPB implicates different processes such as production of phytohormones (usually indole-acetic acid; IAA) that stimulates root growth; qualitative change of flavonoids pattern secreted for the plant; solubilization of non-available nutrients (mainly re-fixation of exogenously applied phosphorus), among others (Medeot et al., 2010). In this section, we summarize the knowledge about bacteria that promote the symbiotic relationship between legumes and rhizobia (from now, the symbiotic enhancer), and the mechanisms involved in this phenomenon. The effect of other microorganisms, such as micorrhizal fungi is not discussed.

Probably the most studied bacterial consortium is the rhizobia-azospirilla one. Azospirilla species are being used as seed inoculants under field conditions for more than a decade (Dobbelaere et al., 2001; Puente et al., 2009). The positive effect of *Azospirillum* in the nodulation and nitrogen fixation by rhizobia on several forage legumes was early reported (Yahalom et al., 1987). Since then, many works have been done and mostly are summarized in Bashan et al. (2004). It proven that the combined inoculation with rhizobia and azospirilla increases the shoot length and weight, root hairs number, root diameter, the main- and total-root nodule number and the percentage of infected root hairs, thus resulting in increased legume yields (Cassán et al., 2009). Worldwide, salinity is one of the most important abiotic stresses that limit crop growth and productivity. It was shown that the rhizobia-azospirilla co-inoculation significantly reduces the negative effects of abiotic stresses (such as caused by irrigation with saline water) on root development and nodulation (Dardanelli et al., 2008).

Under stress conditions, such as drought, salinity, S-deficient or heavy metal (HM)-contaminated soils, several associations between plants and beneficial bacteria showed a defensive response and an increased yield (Anandham et al., 2007; Dary et al., 2010; Fuentes-Ramírez & Caballero-Mellado, 2005; Han & Lee, 2005). However, the physiological mechanism

involved in stress mitigation is still unknown (Figueiredo et al., 2008; Furina & Bonartseva, 2007).

### 3.2 Enhancing the legume – Rhizobia symbiosis by co-inoculation: Modes of action

Many evidences have been accumulated showing that co-inoculation with beneficial microorganisms, having different mechanisms of plant-growth promotion, have additive or synergistic effect on plant growth and crop yield (Table 2). Diverse mechanisms are implicated in the co-inoculation benefits and some of them have been discussed in Barea et al. (2005).

Legume	Bacterial system	Increase (%) compared to single rhizobial inoculation	Experiments done in	Proposed mechanism of action	Reference
Soybean ( <i>Glycine max</i> )	<i>B. japonicum</i> - <i>Serratia</i> spp.	50 in NN; 30 in SDW; 32 in RDW	Greenhouse	Production of LCO- analogue	Bai et al., 2002a Bai et al., 2002b
		40 in NN under sub-optimal temperature	Laboratory	Unknown	Zhang et al., 1996
	<i>B. japonicum</i> - <i>B. cereus</i>	10 in SDW	Field	Unknown	Bullied et al., 2002
	<i>B. japonicum</i> - <i>S. proteamaculans</i> / <i>B. subtilis</i>	12 in SDW; 10 in P-uptake	Greenhouse (saline stress)	Limited Na-uptake	Han & Lee, 2005
	<i>B. japonicum</i> - <i>A. brasilense</i>	47 in NN	Laboratory	Production of IAA, GA3 and Zeatin	Cassán et al., 2009
	<i>B. japonicum</i> - <i>A. brasilense</i>	16-40 in RDW; 200-700 in total RL	Laboratory	Unknown	Molla et al., 2001a
		30 in NN	Greenhouse	Production of plant hormones	Molla et al., 2001b
	<i>E. fredii</i> - <i>Chryseobacterium balustinum</i>	56 and 44 in SDW; 100 and 200 in RDW; 155 and 286 in NN under non-saline and saline conditions respectively	Laboratory (saline stress)	Unknown	Estevez et al., 2009
	<i>B. japonicum</i> - <i>P. putida</i>	40 in SDW; 80 in NN; 45 in RDW	Laboratory	P-solubilization and production of siderophores	Rosas et al., 2006

Legume	Bacterial system	Increase (%) compared to single rhizobial inoculation	Experiments done in	Proposed mechanism of action	Reference
Common bean ( <i>Phaseolus vulgaris</i> )	<i>R. tropici/etli</i> - <i>A. brasilense</i>	18-35 and 20-70 in RDW; 29 and 28 in SDW under non saline and saline conditions, respectively	Hydroponic (saline stress)	Production of flavonoid-like compounds	Dardanelli et al., 2008
	<i>R. etli</i> - <i>C. balustinum</i>	35 in SDW; 35 in NN under non-saline conditions; and 39 in SDW; 63 in RDW under saline conditions	Laboratory (saline stress)	Unknown	Estevez et al., 2009
	<i>R. tropici</i> - <i>Paenibacillus polymyxa</i>	50 in NN; 40 in N uptake in non-drought stress	Greenhouse (drought stress)	Unknown	Figuereido et al., 2008
	<i>Rhizobium</i> spp. - <i>A. brasilense</i> / <i>B. subtilis</i> / <i>P. putida</i>	30 in NN; 20 in SDW; 30-45 in RDW	Greenhouse (two levels of P-fertilization)	IAA production or 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity	Remans et al., 2007
	<i>Rhizobium</i> spp. - <i>A. brasilense</i>	70 in NN	Hydroponic	IAA production	Remans et al., 2008a
		30 total yield	Field	IAA production	Remans et al., 2008b
	<i>Rhizobium</i> spp. - <i>P. fluorescens</i> / <i>A. lipoferum</i>	25 in NN; 13 in SDW; 74 in seed yield	Field	P-solubilization; auxin and siderophores production	Yadegari et al., 2010

Legume	Bacterial system	Increase (%) compared to single rhizobial inoculation	Experiments done in	Proposed mechanism of action	Reference
Chickpea ( <i>Cicer arietinum</i> )	<i>Rhizobium</i> spp - <i>Pseudomonas</i> / <i>Bacillus</i> spp.	20 in SDW; 30-120 in RDW	Greenhouse	Production of flavonoid-like	Parmar & Dadarwal, 1999
	<i>Mesorhizobium</i> sp. <i>Cicer</i> - <i>Pseudomonas</i> spp.	70 in NN; 30 in SDW, 30 in N-uptake	Laboratory	Unknown	Goel et al., 2002
		1,2-1,86 in NN; 1,3-2,11 NFW; 1-2,93 in PDW	Laboratory	IAA production	Malik & Sindhu., 2011
	<i>Rhizobium</i> - <i>B. subtilis</i> / <i>megaterium</i>	18 in SDW; 16-30 in RDW; 14 in total biomass yield in field	Laboratory and Field	N-fixation by <i>B. subtilis</i> or/and P-solubilization by <i>B. megaterium</i>	Elkoca et al., 2008
	<i>M. ciceri</i> - <i>Azotobacter chroococcum</i>	15 in NN; 25 in P-soil availability	Field (two levels of N-fertilization)	Unknown	Qureshi et al., 2009
	<i>M. ciceri</i> - <i>Pseudomonas</i> sp/ <i>Bacillus</i> sp.	20 in PDW; 30 in NN; 100 in P-uptake	Field	P-solubilization by PGPB	Wani et al., 2007
Peanut ( <i>Arachis hypogaea</i> )	<i>Thiobacillus</i> sp. - <i>Rhizobium</i> sp.	50 in PDW; 80 in NN	Greenhouse (S-deficiency) and Field	S-oxidation	Anandham et al., 2007
Clover ( <i>Trifolium repens</i> )	<i>R. leguminosarum</i> bv. <i>trifolii</i> - <i>P. fluorescens</i>	20 in SDW; 100 in NN	Laboratory	Production of B-group vitamins	Marek-Kozaczuk & Skorupska, 2001
	<i>R. leguminosarum</i> bv. <i>trifolii</i> - <i>Delftia</i> sp.	50 in SDW and 80 in nodulation rate	Laboratory	IAA production	Morel et al., 2011
Altramuz ( <i>Lupinus luteus</i> )	<i>Bradyrhizobium</i> sp. - <i>Pseudomonas</i> sp./ <i>Ochrobactrum cytisi</i>	66 in SDW and 20-40, 25, and 30-50 decrease in Cd, Cu and Zn - accumulation in roots, respectively	Field (Heavy metal contaminated soil)	Phyto-stabilization: Biosorption of heavy metals by bacterial biomass	Dary et al., 2010
Alfalfa ( <i>Medicago sativa</i> )	<i>S. meliloti</i> - <i>Delftia</i> sp.	10 in SDW; 30 in nodulation rate	Laboratory	IAA production	Morel et al., 2011

Legume	Bacterial system	Increase (%) compared to single rhizobial inoculation	Experiments done in	Proposed mechanism of action	Reference
Galega ( <i>Galega orientalis</i> )	<i>R. galegae</i> bv. <i>orientalis</i> - <i>Pseudomonas</i> spp.	70 in SDW; 60 in RDW; 30 in NN; 44 in N-uptake	Greenhouse	Production of IAA and/or cellulase by <i>Pseudomonas</i> spp.	Egamberdieva et al., 2010
Vetch ( <i>Vicia sativa</i> )	<i>R. leguminosarum</i> bv. <i>viciae</i> - <i>A. brasilense</i>	30 in SDW	Greenhouse	IAA production and increased root secretion of flavonoids	Star et al., 2011
		nod gene induction and decreased in indoles content	Hydroponic		
Pea ( <i>Pisum sativum</i> L. cv. Capella)	<i>R. leguminosarum</i> bv. <i>viciae</i> - <i>P. fluorescens</i>	1,3 in Pea DW; 0,5-0,69 in plants with disease	Greenhouse ( <i>Fusarium oxysporum</i> infected soils)	Antifungal activity by production of siderophores	Kumar et al., 2001
	<i>R. leguminosarum</i> - <i>B. thuringiensis</i>	84 times in NN; 15 in SDW; 15 in RDW	Laboratory and greenhouse	Unknown	Mishra et al., 2009
Lentil ( <i>Lens culinaris</i> L.)	<i>R. leguminosarum</i> - <i>B. thuringiensis</i>	73 in NN; 5 in SDW; 10-30 in RDW	Laboratory and greenhouse	Unknown	Mishra et al., 2009
Pigeon pea ( <i>Cajanus cajan</i> )	<i>Rhizobium</i> sp.- <i>Bacillus</i> spp.	50 in PFW; 300 in NN	Greenhouse (sterile soil)	Cross-utilization of siderophores produced by <i>Bacillus</i> sp. and <i>Rhizobium</i>	Rajendran et al., 2008
	<i>Rhizobium</i> sp. - <i>P. putida</i> / <i>P. fluorescens</i> / <i>B. cereus</i>	73 in NN; 30 in grain yield	Greenhouse	Unknown	Tilak et al., 2006
Mung bean ( <i>Vigna radiata</i> L.)	<i>B. japonicum</i> - <i>P. putida</i>	20 in total biomass; 48 in NN	Greenhouse	Reduced ethylene production	Shaharoon et al., 2006

Table 2. Ten years of studies on legume co-inoculation (2001-2011). Increase in legume symbiotic parameters and yield by co-inoculation compared to single-inoculation with rhizobia. Abbreviations are as follows: RDW: root dry weight; SDW: shoot dry weight; RL: root length; NN: nodule number; NFW: Nodule fresh weight; PDW: plant dry weight; PFW: plant fresh weight.



Probably, the most reported mechanism that explains the improved rhizobia-legume association by other PGPB is the production of plant-hormones (phytohormones), such as gibberellic acid (GA3) or auxin-type phytohormones (mainly indole-3-acetic acid; IAA; Beattie, 2006). That is the case for *Pseudomonas* (Egamberdieva et al. 2010; Malik & Sindhu, 2011) and *Azospirillum* (Cassán et al., 2009; Dobbelaere et al., 2001; Okon, 1994; Perrig et al., 2007). For information about IAA production and effects, we recommend Baca & Elmerich (2007) and Spaepen et al. (2007). However, the main mechanism involved in improved rhizobia-legume association is still under investigation (Dobbelaere & Okon, 2007). It might be possible that multiple mechanisms, rather than only one are acting. This is known as the "Additive Hypothesis" (Bashan et al., 2004; Bashan & de-Bashan, 2010).

Many other signal molecules or analogues involved in plant-rhizobia communication, different than phytohormones but produced by the non-rhizobial co-inoculant strain, have been implicated in the rhizobia-plant association (Lucas-Garcia et al., 2004b; Mañero et al., 2003). Some direct evidence suggests that the presence of *Pseudomonas* spp. (Parmar & Dadarwal, 1999) and *Azospirillum* spp. cells (Burdman et al., 1996; Dardanelli et al., 2008, Volpin et al., 1996) induce the synthesis of flavonoids by roots of chickpea, common bean and alfalfa, in experiment of co-inoculation with rhizobia. Interestingly, it is not strictly necessary the presence of the bacteria, the application of bacteria-free exudates of symbiotic enhancers to the root exert similar effect that during bacterial-co-inoculation (Molla et al., 2001b). For example, the application of NFs analogues produced by *Serratia proteamaculans* 1-102 promotes soybean-bradyrhizobia nodulation and soybean growth (Bai et al., 2002b). The list of metabolites produced by symbiotic enhancers might become bigger: vitamins that may supplement the nutritional requirement of rhizobia (Marek-Kozaczuk & Skorupska, 2001); hydrolytic enzymes that assist during rhizobial penetration in the root hair, or attack phytopathogenic fungi (Egamberdieva et al., 2010; Sindhu & Dadarwal, 2001; Sindhu et al., 2002); or P-solubilizing acids that increase phosphorus availability (Elkoca et al., 2008). However, in most cases the mechanism underlying the plant growth promotion by co-inoculation is unknown (Bullied et al., 2002; Goel et al., 2002; Lucas García et al., 2004a, 2004b; Vessey & Buss, 2002).

### 3.3 Increasing crop yield by co-inoculation

On average, an increase of 4-5% in crop yield has an important impact in agricultural production. The data obtained in different growth-systems (gnotobiotic laboratory conditions, hydroponics, greenhouse and field) shows that co-inoculation produces a major increase in legume yield compared with single inoculation (Table 2), overwhelming the agronomic expectations.

Inoculation and co-inoculation experiments must be done in field to provide a realistic assessment of the performance of a living-formulation in practical farming conditions. Table 2 shows examples of legume co-inoculation in field experiments. An increase of 74% in seed yield was detected when *Phaseolus vulgaris* was co-inoculated with *P. fluorescens* or *A. brasilense* compared with single-inoculation with *Rhizobium* spp. (Yadegari et al., 2010). As well, 14% total biomass chickpea yield was detected during co-inoculation with P-solubilizing *Bacillus* isolates compared with single-inoculation with *Rhizobium* sp (Elkoca et al., 2008). Vast areas of agricultural land are not appropriated for cropping because the soil has P-deficiency and the co-inoculation of legumes with rhizobia and P-solubilizing bacteria might supply nitrogen and phosphorus to these poor lands. The examples above provided show a huge increase in yield

during co-inoculation in field experiments, pointing the economically relevance of co-inoculation practices in countries with high pulse crop production.

Bacteria	Target crop	Formulation	Yield increase (%) <sup>a</sup>	Reference
Rhizobia - <i>B. subtilis</i>	Soybean; peanuts; dry beans	Co-inoculant <sup>b</sup>	4-6	www.histicknt.com
Rhizobia - <i>A. brasilense</i>	Soybean; peanut; pea; vetch	Co-inoculant	8-30	www.intxmicrobials.com
Rhizobia - <i>A. brasilense</i> - <i>P. fluorescens</i>	soybean	Co-inoculant	5-10	www.inoculantespalaversich.com
Rhizobia - <i>A. brasilense</i>	soybean	Co-inoculant	10	www.nitrosoil.com.ar
Rhizobia - <i>B. megaterium</i> - <i>Saccharomyces cerevisiae</i>	All legumes <sup>c</sup>	Co-inoculant	Undeclared	www.iabiotec.com
<i>B. megaterium</i>	All crops <sup>d</sup>	Inoculant <sup>e</sup>	10	www.rajshreesugars.com
			Undeclared	www.manidharmabiotech.com
P-solubilizing bacteria (genus undeclared)	All crops	Inoculant	10-15	www.gsfclimited.com
<i>Frateuria aurantia</i>	All crops	Inoculant	10-20	www.manidharmabiotech.com
P-solubilizing bacteria (genus undeclared)	All crops	Inoculant	20-30	www.varshabioscience.com
<i>Delftia acidovorans</i>	Canola ( <i>B.napus</i> )	Inoculant	Undeclared	Banerjee & Yesmin, 2004 www.brettyoung.ca

<sup>a</sup> - compared to single inoculation

<sup>b</sup> -the formulation contains both rhizobia and non-rhizobial PGPB in the same package

<sup>c</sup> - recommended for all kind of legumes

<sup>d</sup> - recommended for many crops, including legumes

<sup>e</sup> - the formulation does not contain rhizobia, but it can be used with rhizobial-formulation

Table 3. Some available commercial formulations (containing two PGPB) for legume crops.

Note: mycorrhiza and bio-control bacteria are not included in this list.

Chickpea is the most largely produced pulse crop in India accounting for 40% of total pulse crops production, being the leading chickpea producing country in the world. India annually produces around 6 Million tons of chickpea and contributes of approximately 70% in the total world production. On the other hand, Brazil is the world leader in dry bean production (3.3 Million ton), followed by India (3.0 Million ton) and China (1.9 Million ton). All these countries belong to "the BRICs". In economics, BRIC is a grouping acronym that refers to Brazil, Russia, India and China, which are considered to be at a similar stage of newly advanced economic development. The BRIC thesis, by Goldman Sachs, recognizes that Brazil, Russia, India and China have changed their political systems to embrace global capitalism, and predicts that China and India, respectively, will become the dominant global suppliers of manufactured goods and services, while Brazil and Russia will become similarly dominant as suppliers of raw materials. In this scenario, of countries with growing world economies and important production and consumption of pulses, the development of new formulations based in bacterial consortiums are being encouraged. However, a major constraint for exploiting living-formulation technologies has been that most farmers are not aware of the technology and its benefits.

### 3.4 New formulations: The use of bacterial consortium

Some bacterial symbiotic enhancers are promising microorganisms that would be used for the design of new formulations. These formulations could contain different bacteria in one pack, ready for direct placing in the seed at planting. However, some manufacturers also produce formulations that do contain non-rhizobial PGPB, but that can be mixed with rhizobial-formulation at the moment of planting. Information on both kinds of formulations is provided in Table 3.

Despite the great progress and the increasing interest in mixed formulations for legumes inoculation, there are few commercial products with different bacteria. Most of these products are based on *Bacillus* spp. *Azospirillum*-based inoculants are also abundant in the market, but most of them are available for non-legumes crops (Figueiredo et al., 2010). Most commercially available biofertilizers are biopesticides and biofungicides, but they are not described in this chapter.

## 4. Concluding remarks

The doubling time world's current growth is 54 years and we can expect the world's population to become 12 billion by 2054. This demographic growth has to be accompanied by an increase in food production. Thus, the humanity has to face a new challenge, by doing a good use of soils (Fedoroff et al., 2010; Godfray et al., 2010) and developing new technologies (Pretty, 2008), mainly based in eco-friendly microorganisms that control pest and improve plant growth. In such scenario, the use of biofertilizers, rhizobia or consortium of plant-beneficial microbes (rhizobia and symbiotic enhancers) in formulations provides a potential solution. The data showed in this chapter support that the design of new formulations with cooperative microbes might contribute to the growth improvement of legumes. The co-inoculation has a positive effect in growth stimulation of legume crops; however, we believe it is necessary to continue studying this subject.

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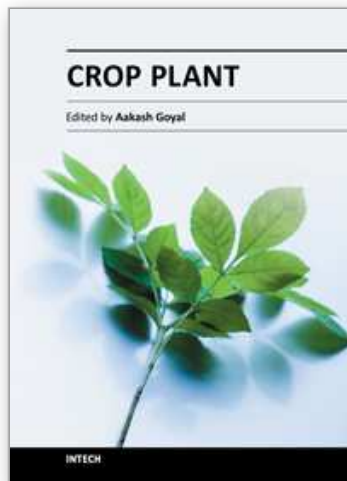
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This book provides us a thorough overview of Crop Plant with current advance in research. Divided into two section based on the chapters contents. Chapter 1 provides information about markers and next generation sequencing technology and its use. Chapter 2 is about how we can use Silicon for Drought tolerance. Chapter 3 is to deal with the major problem of rising CO<sub>2</sub> and O<sub>3</sub> causing environmental pollution. Chapter 4 covers the phenomena of RNAi and its use, application in crop science. Chapter 5 is a review for boron deficiency in soils and how to deal with it for better crops. Chapter 6-10 provide some information regarding recent works going on in crop science.

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