19

Signals in Soybean´s Inoculants

Nápoles María C[1], Gómez Gretel[1], Costales Daimy[1], Freixas JA[1], Guevara E[2], Meira S[2], González-Anta G[3] and Ferreira A[3]
[1]*Instituto Nacional de Ciencias Agrícolas*
[2]*Instituto Nacional de Tecnología Agropecuaria,*
[3]*Rizobacter Argentina SA*
[1]*Cuba*
[2]*Argentina*

1. Introduction

Legumes are an important component of all agricultural systems because of the nitrogen fixation provided by their root nodule bacterial symbiont, rhizobia. Among the legumes, the Soybean, also classed as an oilseed, is pre-eminent for its high (38-45%) protein content. Production of high-quality, protein-rich food is extremely dependent upon availability of sufficient N. Even though N is among the most abundant elements on Earth, it is the critical limiting element for growth of most plants due to its unavailability (Graham & Vance, 2000). Plants acquire N from two principal sources: the soil, through commercial fertilizer, manure, and/or mineralization of organic matter; and the atmosphere through symbiotic N_2 fixation. Symbiosis between leguminous plants and rhizobia, under conditions of nitrogen limitation, leads to the development of new plant organs, the N2-fixing nodules, which are usually formed on roots but also on stems in a few plants after the diazotroph and its host positively recognize each other. The root nodule is the site where N2 gas is reduced to ammonia, which is assimilated into amino acids; these are then used to synthesize other nitrogen-containing compounds. Inside the nodule the differentiated form of rhizobia, the bacteroids, fix molecular nitrogen, which is then used by the plant partner. Effective, nitrogen-fixing rhizobium–legume symbiosis requires an intricate molecular dialogue between the two interaction partners before and during invasion by the microsymbiont (Broughton et al., 2000; Perret et al., 2000). Host specificity is determined by several factors. From the bacterial side, the main signalling molecules are Nod factors, surface polysaccharides and secreted proteins. This chapter outlines an overview of the role of major determinants and signals in the symbiotic process, as well as some results obtained by the authors using *Bradyrhizobium* induced inoculants and its effect on soybean development.

2. Nod factors

The symbiotic interaction starts when the bacteria colonize the root surface and induce curling of the root hair tips (Long, 1996; Schultze et al., 1994). This is followed by cell wall invagination and the formation of an infection thread that grows within the root hair. The infection thread traverses the outer cell layers to reach the nodule primordium, which is

initiated by the reactivation of differentiated cells of the root cortex for division. Within the infection thread the rhizobia multiply but remain confined by the plant cell wall. As the primordium develops to a nodule, bacteria are released from the tip of the infection thread by endocytosis and differentiate into bacteroids surrounded by the peribacteroid membrane. That symbiotic interaction involves an exchange of complex molecular signals that confer specificity. Legume roots and seeds exude different substances: sugars, amino acids, dicarboxilic acids and various aromatic compounds such as some flavonoids (Brencic & Winans, 2005), in mixtures that differ between species. Rhizobia respond to these because they have one or more *nodD* genes, which encode regulator proteins that activate the other *nod* genes when they interact with appropriate plant signal compounds. Once activated, the *nod* genes direct the synthesis of Nod Factors (NF), a family of lipochitin oligomers (LCO), which acting as morphogens, initiating the nodulation program of the host plant (Schultze & Kondorosi, 1998; D'Haeze & Holsters, 2002). Structurally, all Nod factors are based on short chains of β 1,4-linked N-acetylglucosamine residues. The distal or non-reducing glucosamine residue is N-acylated. This common lipo-chitooligosaccharide core (produced by the enzymes encoded by the *nodABC* genes) may be modified by various specific substituents on the distal or reducing sugars. These modifications are governed by additional *nod* genes. Rhizobial species and genotypes differ in their complement of *nod* genes and in allelic forms of shared *nod* genes, and these differences lead to predictable differences in the structure of the Nod factors. NFs from several rhizobial species have been characterized and their structures have been determined. *Bradyrhizobium´*s LCO are produces mainly in response to flavonoids. For instance, the isoflavones daidzein and genistein, the main components present in soybean root extracts, are responsible for inducing the *nod* genes of *Bradyrhizobium japonicum* (Kosslak et al., 1987). Nodulation leads to the colonization of plant cells by invading bacteria. Although many host plants and effective rhizobia have the ability to enter into symbiosis with more than one partner, only certain combinations of symbionts result in the formation of nitrogen-fixing nodules. Ineffective associations lead to empty or nonfixing bacteroid-containing nodules. Specificity among compatible partners minimizes the chances of infection by pathogens and the formation of ineffective associations that are detrimental to both symbionts. Experimental evidence suggests that the progression of invasive rhizobia towards nodule primordia is challenged at various "doors". Codes contained in molecular signals open these checkpoints. During the initial phases of nodulation (root hair curling and bacterial entry), these codes are given by flavonoids (from the plant) and Nod factors (from the bacteria). In both cases, NodD proteins are the chief interlocutors of molecular traffic in the rhizosphere. Many studies have implicated Nod factors as a possible candidate in the host specificity of rhizobia, inducing several responses in plant until complete nodule (Vijn et al., 1993; Stokkermans & Peters, 1994; Heidstra et al., 1997; Spaink, 2000; Geurts et al., 2005). They invoke multiple physiological responses in the host, such as: root hair deformation (Lhuissier et al., 2001), induction of nodulin genes essential for infection thread formation and cortical cell division (Schlaman et al., 1997). Other authors have been found that Nod factors reduces the salicylic acid (SA) level in roots and this might help in the suppression of host defense responses, thus ensuring successful infection by rhizobia (Martinez-Abarca et al., 1998). Similar decreases in SA level in the leaf tissues occurred when soybean plants were sprayed with Nod factor (Prithiviraj et al., 2000). On the other hand, Nod factors from *Bradyrhizobium* have been shown to enhance seed germination and early seedling growth of its target plant, soybean, but also enhances these parameters in non-target plants from

diverse botanical families (Prithiviraj et al., 2003; Miransar & Smith, 2009). This way, nodulation factors or LCO have been considering the key to open door legumes, the first and main determinant (Relic et al., 1994, Long, 1996; Broughton et al., 2000; de Haeze et al., 2002; Gage, 2004).

3. Polysaccharides

Together with Nod factors, other bacterial components have been involved in bacterial adhesion, formation and extension of the infection thread, releasing of bacteria into the nodule cells and differentiation into bacteroids. Between these components, polysaccharides have a relevant role. Rhizobia synthesize different classes of polysaccharides: exopolysaccharide (EPS), capsular polysaccharides (KPS), lipopolysaccharides (LPS) and the cyclic glucan. Some of them are secreted to the media, others are exposed on the surface or present in the periplasmic space (Lepek & D´Antuono, 2005). EPS appears to be essential for the successful invasion of indeterminate nodules (Pellock et al., 2000), whereas LPS are involved principally in the formation of determinate nodules, especially during the initiation and elongation of the infection thread (Lerouge & Vanderleyden, 2001; Noel et al., 2000). Both, in determinate and indeterminate nodule formation, the absence of cyclic glucan synthesis affect the invasion capacity of the bacteria (D´Antuono et al., 2005). *Bradyrhizobium japonicum* strain 2143 and two derivative strains are capable of producing three exopolysaccharides that appear to be involved in the efficiency of their symbioses with *Glycine max* (Karr et al., 2000), and *B. japonicum* strain USDA 123 produces two structurally distinct polysaccharides, one when outside the nodule and the second when inside the nodule (An et al., 1995). Additionally, the symbiotic defects of EPS-deficient mutants of *B. japonicum* strain 110*spc*4 are host dependent, differing markedly on the hosts *Glycine max* and *Glycine soja* (Parniske et al., 1994). The results obtained by different groups suggest a possible role in two main points in the process, the bacterial relation with the defense response generated in the plant and the intimate interaction between bacterial and plant cell membranes. Therefore, polysaccharides are critical for the establishment of a productive plant-bacterium symbiosis, what become it in the second determinant of this symbiosis.

4. Protein secretion systems

Besides to previous host specificity determinants described (Nod factors and surface polysaccharides); there is a third class of rhizobial signals that can affect symbiosis between *Rhizobium* and Legume. It consists of secreted proteins. Cells from prokaryotes and eukaryotes alike must transport proteins across the membranes that envelop them (Economou & Dalbey, 2004). In Gram-negative bacteria, as *Rhizobium*, the transport is more complicated due to the presence of two barriers, the inner and outer membrane (IM and OM). Several secretion systems seem to have specialized in mediating *Rhizobium*-legume interactions, with the ability to translocate effector proteins into the host cell cytoplasm as a defining feature. These systems include the T3SS, T4SS and T6SS (Papanikou et al., 2007). T3SS are complex macromolecular structures that span not only the IM and OM, but the host cellular membrane as well. They allow direct translocation of secretion substrates into the host cell cytoplasm. The secretion signal is poorly defined and located in the amino-terminus of secretion substrates (Ghosh, 2004; He et al., 2004). Proteins secreted through rhizobial T3SS are called nodulation outer proteins or Nops. The first secreted rhizobial

protein for which a role in symbiosis could be shown was *Rhizobium leguminosarum* bv. viciae NodO (de Maagd et al., 1989). NodO was detected in spent medium of cultures grown in the presence of flavonoids and expression was found to be NodD dependent (de Maagd et al., 1988). These are also found in promoter regions of genes unrelated to T3SS, such as those involved in the biosynthesis of rhamnose-rich polysaccharides (Marie et al., 2004). This led to the discovery of a complex interplay between the T3SS and surface polysaccharides in the molecular dialogue of the rhizobium–host interaction (Broughton et al., 2006). In *Rhizobium* species strain NGR234 at least six T3SS-secreted Nops have been identified: NopA, NopB, NopC, NopL, NopP, and NopX (Ausmees et al., 2004; Deakin et al., 2005; Marie et al., 2003; Saad et al., 2005; Viprey et al., 1998). Depending upon the legume host, abolition of Nop secretion by NGR234 can improve or block symbiotic interactions. T3SS genes were subsequently identified in *B. japonicum* USDA110, *Sinorhizobium fredii* strains HH103 and USDA257, *Mesorhizobium loti* MAFF303099 and *R. etli* CNPAF512 (Kaneko et al., 2000; Krause et al., 2002; Krishnan et al., 2003; Hubber et al., 2004; de Lyra et al., 2006). In each case, the T3SS affects symbiosis in a host-specific manner. Taking into account that T3SS have previously related to microbe-host interaction could be possible that rhizobia use the exact same secretion mechanisms as their pathogenic counterparts in trying to persuade prospective hosts to allow rhizobial invasion (Fauvart & Michiels, 2008).

5. Inoculants to soybean

Dinitrogen fixation provides more N to the agricultural ecosystems worldwide than the total amount of fertiliser N applied. Soybean plants can fix nitrogen at rates of up to 200 $kg.ha^{-1}.year^{-1}$, eliminating the need for environmentally and economically costly nitrogen fertilizers (Ip et al., 2001). When soybeans are cultivated for the first time, inoculation with bradyrhizobia is essential for high yields. A number of different types of soybean inoculants are available. Advancements in technology have provided inoculant types with higher rhizobia concentrations and more options for planting systems. Due to the importance of nodulation for agriculture, intensive researching is being carried on in this area in order to understand the molecular bases of this process. The new knowledge could be used to obtain more efficient nitrogen fixation process, modification of the host range or increased competitiveness that may influence its capacity to compete in the rhizosphere with other bacteria. Better N2-fixing symbiosis may be brought about by manipulating both rhizobia and plant hosts and by eventually creating an artificial rhizosphere. An important aim is also to improve the symbiotic relationship in suboptimal environmental situations related to environmental stress. Plant nodulation and nitrogen fixation processes in nature are affected by the micro-ecology of the plant rhizosphere. Soil temperature, pH, texture, moisture, salinity, and deficiencies in essential elements inhibit all stages of symbiotic establishment investigated to date (root hair curling, infection thread formation and penetration, nodule formation and function) (Zahran, 1999). The infection and early nodule development processes are most sensitive to stressful environmental conditions. Although combinations of rhizobia and plants may be compatible, nodulation failure can still occur in the field (Robson & Bottomley, 1991). For example, the exudation of flavonoid compounds from clover roots required for *nod* gene induction in *R. leguminosarum* bv. *trifolii* was reduced when the plants were grown at pH<5 (Richardson et al., 1988). The presence of nitrogen in the root rhizosphere also limits the nodulation of legumes (Streeter, 1988), while nitrogen (as ammonia) has been shown to limit the induction of the *nodABC* genes (Dusha et al., 1989). In

the case of soybean, the time between inoculation and onset of nitrogen fixation is delayed by 2–3 d for each degree decrease in temperature from 25°C to 17.5°C. However, when the root zone temperature drops below 17.5°C, the onset of nitrogen fixation is sharply delayed by 7 d for each degree decrease (Zhang et al., 1995). Low temperature was also found to decrease both the biosynthesis of isoflavonoids and the excretion of these signal compounds from plant root cells to the rhizosphere (Zhang et al., 1995). Inoculating soybean with preinduced *B. japonicum* improved soybean nodulation and shortened the time between the onset of nitrogen fixation under low root zone temperature conditions (Smith & Zhang, 1999).

5.1 The culture media

Every organism requires finding all necessary substances for cellular biosynthesis and energy generation during its life. The culture media constitute the micro world of microorganisms in laboratory conditions. The design of a culture medium must answer the exigencies of specific bacteria and the finality we followed with it reproduction, since the composition may influence different microbial physiologic aspects: nutrition, multiplication and primary or secondary metabolites production (Bernal et al., 2002). Decreased legume grain yields, as compared with growth of the same crop with N fertilizer addition, have been reported. This is generally associated with poor-quality inoculants, with low numbers of bacterial cells (Hume & Blair 1992; Singleton et al., 1997; Lupwayi et al., 2000; Catroux et al., 2001; Supanjani et al., 2006). Also, abiotic stress factors can cause poor nodulation in the presence of otherwise compatible symbionts. Early events in the symbiosis such as signal production and excretion, rhizobial attachment, root hair curling, infection thread formation, and nodule initiation, are particularly sensitive to some stresses (Duzan et al., 2004). The fact that commercial inoculants generally compete poorly against indigenous strains (Loh & Stacey, 2003), have incited the continuous work of commercial industrial to provide high quality rhizobial inoculants for agricultural production. In that sense, our group has been working in the obtainment of soybean´s inoculants which contain as base a good culture media to guarantee the optimal multiplication of bradyrhizobia, the specific inducers to assurance the activation of all determinants in the symbiosis success and as result a product with good quality to be translated into agronomical sustainable benefits.

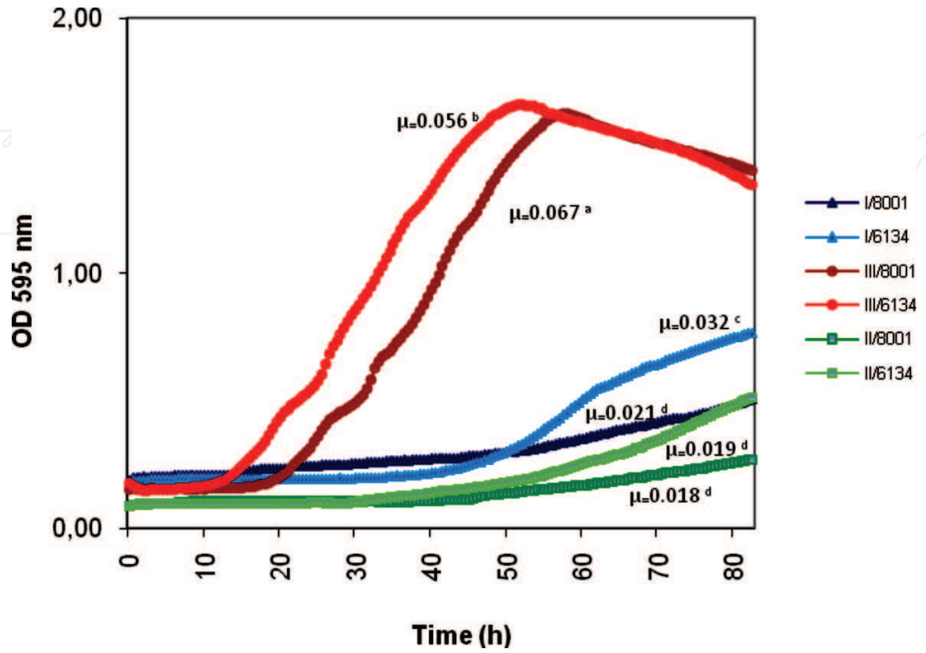
5.1.1 The culture media in multiplication

Our first studies evaluated different culture medium composition on multiplication of two *Bradyrhizobium elkanii* strains (*B. elkanii* ICA 8001 and *B. elkanii* LMG 6134). We use traditional media reported to bradyrhizobia (Vincent, 1970, López, 1990) and a new composition registered as Cuban Patent No. 22 797 (Nápoles, 2002). The strains were cultivated during 6 days at 30ºC and 230 rpm in orbital shaker conditions. The multiplication rate was measured by Bioscreen C (Labsystems, Helsinki, Finlandia), at 595 nm. The optical density was calculated for each time and the growing specific velocity was determined in lineal phase of growing by:

$$\mu = \frac{\ln(OD_2 / OD_1)}{(t_2 - t_1)}$$

The results shown clear differences between media compositions used for the two strains (Nápoles, et al., 2006). Not only cell density, but growth specific rates were higher with the

new medium proposed in both strains (figure 1). The components of a new culture medium for *Bradyrhizobium*, which contain different carbon, nitrogen and other nutrient sources, allow obtaining more cells at the same period of time.



ES = 0.0027, p≤ 0.001, n=5
μ: growth specific velocity

Fig. 1. Growth dynamic of *B. elkanii* ICA 8001 and *B. elkanii* LMG 6134 in three culture media (I and II traditional media, III new composition of culture media)

5.1.2 The culture media in Nod factors induction

Nod Factors biosynthesis by rhizobia is dependent on several factors. Therefore, the composition of the medium in which the rhizobia are grown is likely to affect NF production in a qualitative and quantitative manner. To compare the three culture media on the *nod* genes activation in *Bradyrhizobium elkanii* ICA 8001, we use two ways: β glucuronidase assay and Nod factor production. Triparental mating was carried out using the donor strain DH5α/pGUS and the helper strain HB101/pRK2013 from *E. coli* and the *B. elkanii* wild-type strain as an acceptor as described in (Hahn & Hennecke, 1984). *B. elkanii* conjugation mixtures were plated out on peptone-yeast extract medium with Km and Nal (30μg.ml^{-1}) to select for the *Bradyrhizobium* colonies harbouring pGUS32Km. Quantitative analysis of GUS A activity was then carried out with *p*-nitrophenyl-ß-D-glucuronide (pNPG) as the substrate in microtiter plates and GUS A activity was examined in VERSA max microplate reader (Molecular Devices). To determination of Nod factors profile, the nodulation factors were radioactively labelled and they were isolated by following a slightly modified protocol of (Laeremans et al., 1998). 100μL from *Bradyrhizobium* cultures, growth for two nights, were inoculated in 900μL of each fresh culture medium and the concentration was adjusted to 5x10^8 CFU per medium milliliter. They were pre-incubated to 30°C with agitation, during 1h. Each sample was supplemented with genistein 10μM as inducer and incubated during 2 hours at the same temperature and agitation. After the

induction the isotopic label was carried out adding 125 µL of ^{14}C [2-^{14}C] acetic acid as sodium salt. The cells were labelled for 36h. The nodulation factors were isolated twice with 500µL n-butanol and washed with ethyl acetate. The solution was vacuum-dried and samples were applied on reverse-phase TLC plates (RP-18 F_{254s}, Merck). H_2O/acetonitrile (1:1, vol/vol) was used as the mobile phase. The radioactivity was visualized by autoradiography using Hyperfilm- β max (Amershan Life Sciences) after 4 days of exposure. The results show a correspondence between the *nod* genes expression by ß-glucuronidase activity and by Nod factors production determinate in TLC and autoradiography (Table 1).

Culture Media	Medium I		Medium II		Medium III	
	(A)	(B)	(A)	(B)	(A)	(B)
Miller units	-6.384		1.669		17.676	
	-5.407		1,360		22.209	
	-6.138		1.891		18.433	
	-5.990		1,428		17.831	
	-5.949		1.241		48.96	
	-6.006		1.507		33.619	
	-5.381		1.554		32.685	
	-5.778		1.482		33.222	
	0		1.587		19.037	
	0		1.446		37.121	
AV	**0**		**1.517**		**28.079**	

Table 1. Effect of different medium compositions on ß-glucuronidase activity (A) and Nod factors synthesis (B).

Medium I did not expressed GUS activity and the TLC showed no spots of Nod factors produced. Medium II induced a low enzymatic activity and two or three spots of Nod factors, while Medium III induced a major ß-glucuronidase activity and correspondently a high Nodulation factors production with at least five different structures of this biomolecules (Nápoles, et al., 2003). Then, another important characteristic of a culture medium is the possibility to contain natural substances which induce the *nod* genes in the bacteria to increase the nodulation factors concentration in the inoculum. A positive effect of genistein addition, as a *nod* gene inducer to *B. japonicum* inoculants on soybean grain and protein yield (Zhang & Smith, 1996), nodulation efficiency (Pan et al., 1998), N2 fixation and total N yield at low root zone temperatures (Zhang & Smith, 1997) has been reported.

5.2 The inoculants activity in plants

To corroborate the efficiency of different inocula derivated from culture in different media assays in plants were performanced. In the experimental set-up for *in vitro* evaluation, plants were grown in the plant growth room with a 12h photoperiod (day/night temperature 26ºC/22ºC; relative humidity 70%) as described by (Michiels et al., 1998). Four weeks after inoculation, nitrogen fixation capacity of the inoculated plant was determined by means of the acetylene reduction assay (ARA) using a gas chromatographer (5890 A; Hewlett-Packard, equipped with a "PLOT fused silica" column). Other parameters such as the number of nodules and fresh and dry weights of nodules per plant were determined (Nápoles et al., 2005). The obtained results favor inoculants grown in medium III since not only more nodules were formed, but also a higher ARA was measured (table 2).

Culture medium	Number of nodules per plant	Fresh weight of nodules per plant (g)	Dry weight of nodules per plant (g)	ARA (µmolethylene. plant^{-1}.h^{-1})
Medium I	13.3 c	0.16 c	0.02 b	1.9 c
Medium II	26.3 b	0.21 b	0.05 b	4.1 b
Medium III	55.0 a	0.37 a	0.09 a	7.8 a
Control	0 d	0 d	0 c	0 d
SEx	2.87***	0.03**	0.007***	1.07**

Values followed by the same letter are not significantly different ($p \leq 0.05$).

Table 2. Effect of different media on nodulation and nitrogen fixation.

For experimental set-up of field trials, at harvest (90 days after sowing), plant heigh, number of pods, overall yield and weight of 100 grains were determined in two different soils.

Treatments	Height (cm)	Pod number per plant	100 grains weight (g)	Yield (t.ha^{-1})
Medium II	70.67	30.40	12.0 b	1.26 b
Medium III	76.47	40.87	16.0 a	1.46 a
SEx	1.85 ns	3.29 ns	0.86**	0.04**

Values followed by the same letter are not significantly different ($p \leq 0.05$).

Table 3. Effect of different culture media on soybean yield, analyzed in a red ferralitic, compact and saturated soil.

The superior effectiveness of medium III was corroborated. Table 3 shows a 15.8% yield increase when medium III was applied in the saturated soil type. In the case of unsaturated soil, a 14.4% yield increase was observed (table 4). Clearly, the positive effect of the culture medium on nodulation resulting in higher N2-fixation per plant is reflected by a higher yield in field trials.

Treatments	Height (cm)	Pod number per plant	100 grains weight (g)	Yield (t.ha^{-1})
Medium II	109.92 b	53.40 b	11.75 b	2.08 b
Medium III	114.12 a	66.82 a	15.5 a	2.38 a
SEx	1.05*	2.92**	1.01*	0.07*

Values followed by the same letter are not significantly different ($p \leq 0.05$).

Table 4. Effect of different culture media on soybean yield, analyzed in a red ferralitic, compact and unsaturated soil.

5.3 The inocula optimization

Bradyrhizobium elkanii ICA 8001 is used in Cuba for soybean inoculants production. The employ of *Bradyrhizobium* inoculants have been supplied between 80 and 100% of nitrogen requirement of this crop (Treto et al., 2005). After many years of research, (Pijeira & Treto, 1983; Pijeira et al., 1988) recommended the inoculation of this strain for numerous Cuban and introduced commercial soybean varieties. The culture medium design plays a crucial role for high cell density inoculants obtaining. The presence of specific nutrients in the culture medium, as well as their concentrations determine the good cells functioning and

influence on cellular metabolism, promoting the biomass or others specific metabolites production. On fermentative microbial process it is necessary culture medium and environmental conditions optimization to exploit completely the potential of the selected strains (Parekh et al., 2000). The choice of the adequate statistical tools is very important to save time and resources. Single variable optimization methods are not only tedious, but can also lead to misinterpretation of results, especially taking into account that the interaction between different factors is overlooked (Abdel-Fattah et al., 2005). Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response (Kim et al., 2005; Lee & Gilmore, 2005; Nawani & Kapadnis, 2005; Senthilkumar et al., 2005; Wang & Lu, 2005; Gómez & Batista, 2006). The carbon, nitrogen and phosphorus sources are macronutrients of vital importance on cellular growth and maintenance, for this reason the influence of these compounds individually and their combinations on *B. elkanii* ICA 8001 cellular multiplication was evaluated by using Response Surface Methodology (RSM), a central composite design (CCD) and statistical analysis. The experiments were carried out at orbital shaker level and the cellular growth was expressed in colony forming units per ml of culture sample. The optimization process finished with the obtaining of a new optimized culture medium for *B. elkanii* ICA 8001 cellular multiplication. A value of 10^{11} CFU/ml with the optimum medium was reached, increasing in one level the value of viable cells obtained with the reference medium (figure 2). The new concentrations varied remarkable respect to reference medium, carbon source 2 concentrations increased in a 43%, phosphorus quantity increase in a 35% and carbon source 1 concentrations in a 5.2%, nevertheless the nitrogen concentration decreased in a 30%. This increase on carbon 2 source concentration promoted the growth of this bacteria, explained by its high contribution of assailable compounds to the cells, in the same way the phosphorus sources increasing, determinating a higher biomass production. The CCD method was efficient, because the optima concentrations of the three independents variables were determined with only 17 trials, which allowed the increasing of bacterial growth in a 10%. Besides, the design evaluated the concentrations interaction effect on the variable response.

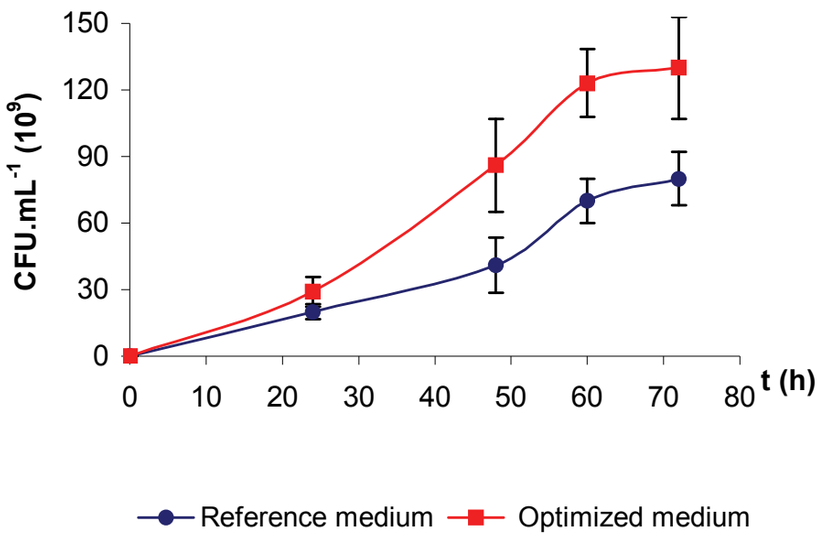


Fig. 2. Time course of cell density of *Bradyrhizobium elkanii* ICA 8001 in Medium III (Reference) and optimized medium, at 28 ± 2º C and 100 rpm.

Numerous are the works that demonstrated the Central composite design affectivity in the culture media optimization for microorganisms. (Lee et al., 1998) used this design for cholesterol oxidase enzyme production by *Rhodococcus equi* no. 23 and (Bhosale & Gadre, 2001), for the growth and carotenoids production by mutant 32 of *Rhodotorula glutinis*. The combination of complete and fractional designs is an effective tool in the optimization process, because they complement each other and permit to achieve significant response variables enhancing. For example, (He et al., 2004) employed a fractional factorial design to evaluate the effect of glucose, pectin, soybean cake extract, casein, corn flour, ammonium sulphate, sodium bicarbonate concentrations and initial pH on the growth of the probiotic strain *Clostridium butyricum*. Then the optimal concentrations of these compounds were found by a CCD. After 24 h of fermentation in the optimized culture medium a viable bacteria population of 10^9 CFU/ml was reached.

It is possible also to optimize a culture medium composition in order to achieved higher cell concentration, as well as, higher Nod factor production (figure 3).

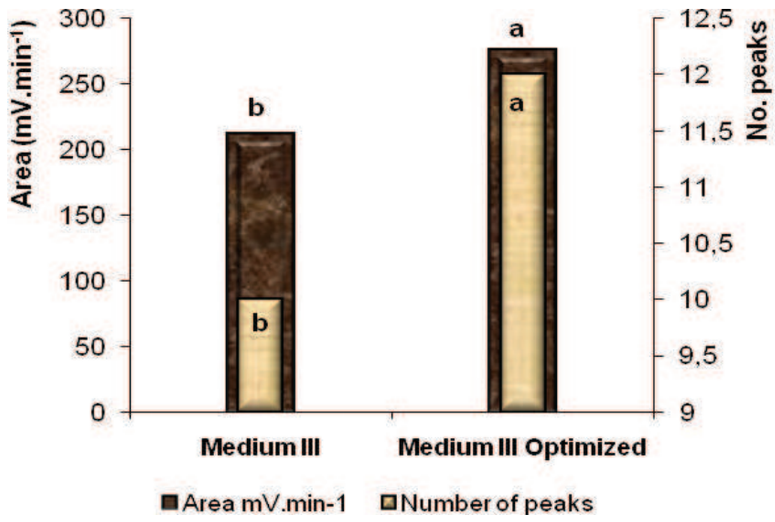


Fig. 3. Nodulation factor values of area under curve and number of peaks for Medium III before and after optimization. (Duncan, $p<0,05$). $SE_{picks}= 0.036$; n=3. $SE_{Area}= 36,45$; n=3.

In this sense, each inducer component of medium III and it concentration was optimized using similar methods to obtain a higher Nod factor production, this time evaluated by High Performance Liquid Chromatography (HPLC). The Nod factors purified were dissolved in 100% acetonitrile and then injected into a normal phase column Ultropac TSK OH-120 5um LKB with dimensions of 4.6 x 250 mm. The flow rate 1 $mL.min^{-1}$; as solvent were used: acetonitrile (A): water (B); the detector: an UV spectrophotometer at 206 nm and a cell of 10 mm, the Gradient in a Knauer pump: 0/0 10/0 70/20 t/%B and with an Injection of 250 μL in 100% acetonitrile. The run time in all cases was 70 minutes. The chromatographic profile of the Nod factors was analyzed according to the number, distribution and relative intensity of the obtained peaks. The number of peaks, corresponding to Nod factors and their area were higher when medium was optimized. The use of the optimization strategy allowed the obtaining of a new culture medium, with a nutrients and inducers balance, which guarantied the obtaining of high density cellular inoculums and the production of high Nod factors concentration. The fact that this

composition medium improves the growth and induction of this slow-growing rhizobial bacterium makes the *Bradyrhizobium* inoculants production a cheaper and efficient fermentative process.

5.4 Inoculants induced and the effect on drought stress

The soybean is a crop mainly grown under rain fed conditions although irrigation is increasingly being used. As with other grain legumes, soybean is very sensitive to drought stress which leads to reduced yield and seed quality (Bosniols et al., 1986; Frederick et al., 2001; Purcell et al., 2004). Negative effects of water stress on growth, photosynthesis, and photoassimilate translocation in soybean were demonstrated by (Ohashi et al., 2000) and (Fulai et al., 2004). The symbiosis process is also negatively affected by water stress, leads to decreased nodule formation, reduced nodule size and N fixation (Serraj et al., 1999; King and Purcell, 2001; Serraj, 2003; Streeter, 2003; Tajima et al., 2004). Negative effects of water stress on growth, photosynthesis, and photoassimilate translocation in soybean were demonstrated by (Ohashi et al., 2000) and (Fulai et al., 2004). The symbiosis process is also

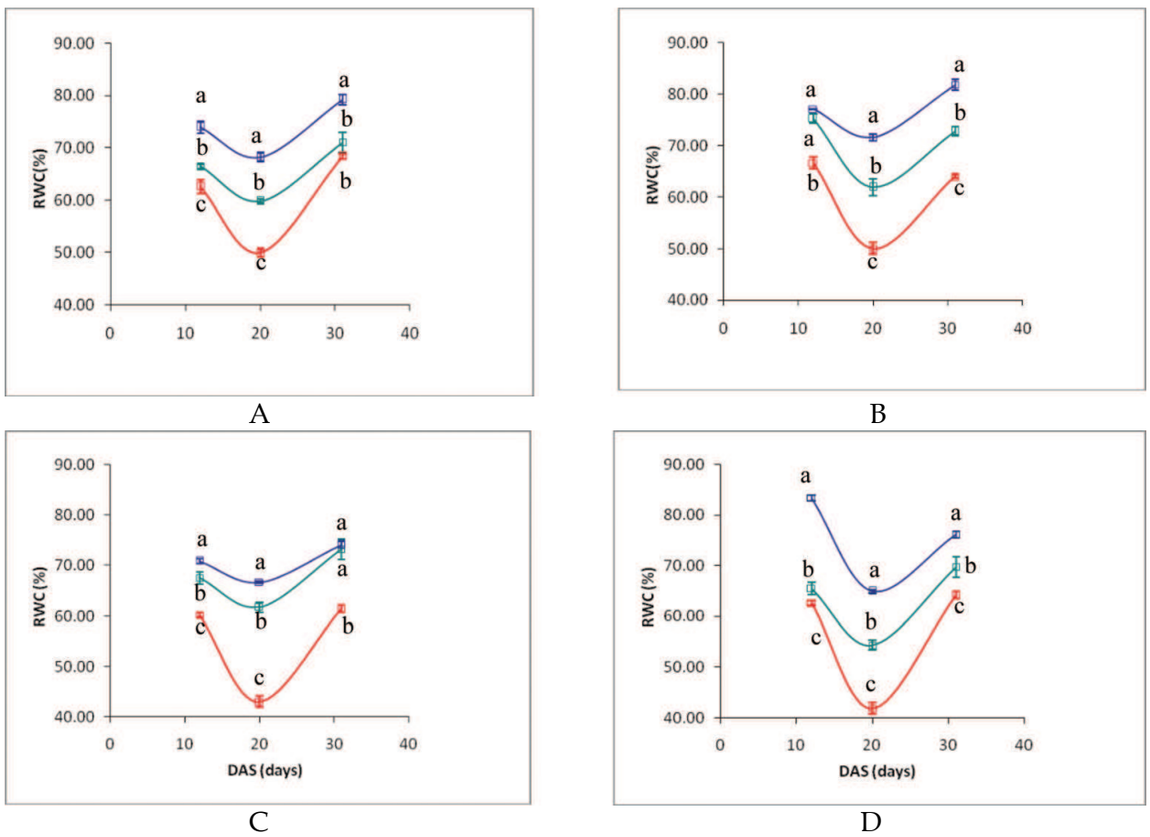
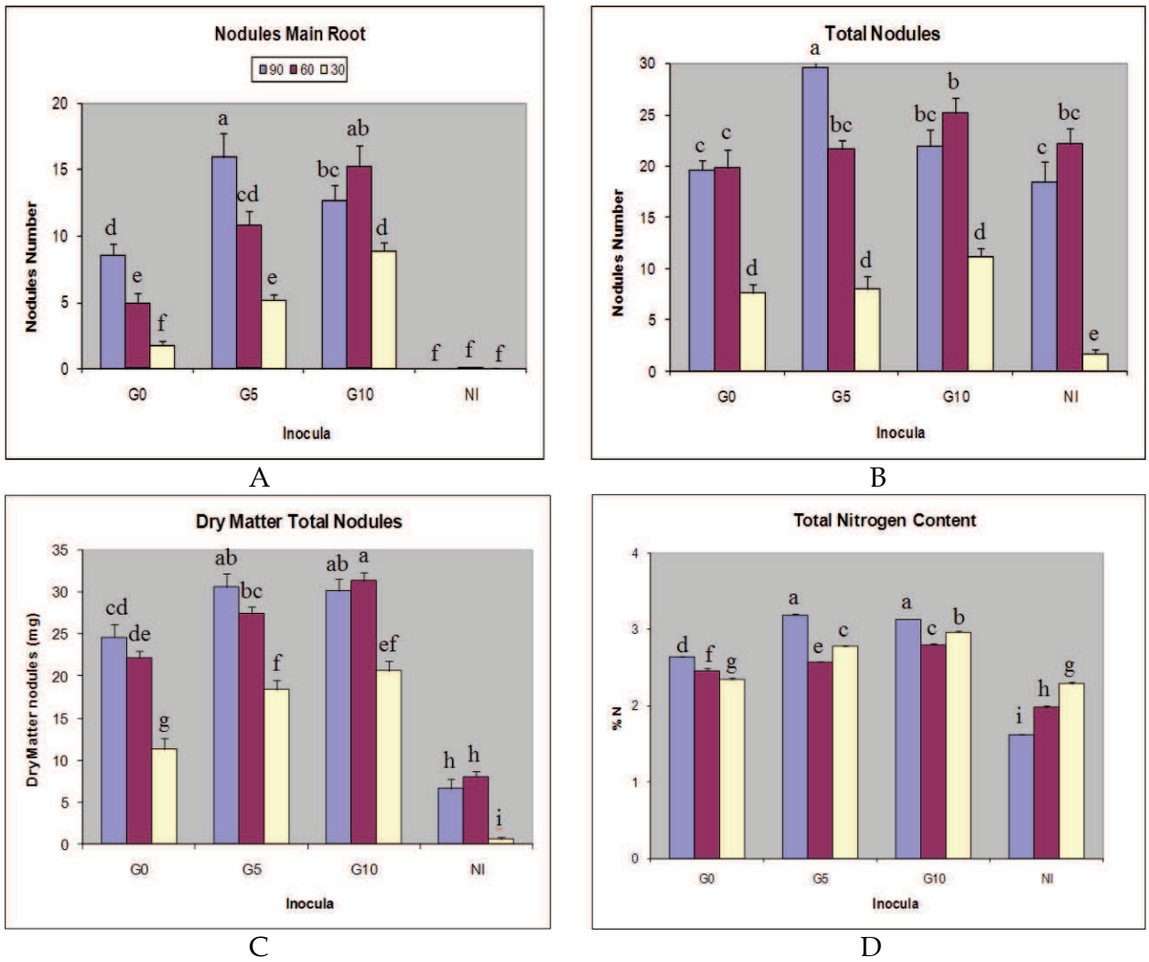


Fig. 4. Relative Water Content in plants inoculated (A: medium without induction, B and C: medium supplemented with genistein 5 and 10 μM, respectively) and non inoculated (D) with three irrigation levels (red: 30% field capacity, green: 60% field capacity, blue: 90% field capacity) during 12, 20 and 31 days after sowing (DAS). Vertical bars indicate ± standard error (SE). Treatments with different letters are significantly different, ANOVA (p<0.05) $LSD_{0.05}$.

negatively affected by water stress, leads to decreased nodule formation, reduced nodule size and N fixation (Serraj et al., 1999; King and Purcell, 2001; Serraj, 2003; Streeter, 2003; Tajima et al., 2004).

The isoflavone genistein have been recognized as a powerful inducer of Nod factors production by *Bradyrhizobium* and its addition to inocula has been shown to increase nodule number and promote soybean nitrogen (N) fixation at low temperatures. The impact of lipochitinoligosaccharids spray application on the physiology and productivity of water stressed soybean plants was evaluated by (Atti et al., 2005). Foliar application of Nod factors affected plant physiological activity, increased flower and pod numbers, and accelerated leaf senescence of soybean plants under water stress. Our study looks for answers about the possible role of genistein in countering the stress on nodulation produced by water deficit in soybeans.



G0: medium without induction, G5 and G10: medium supplemented with genistein 5 and 10 μM, respectively. NI: control non-inoculated.
Treatments with different letters are significantly different, ANOVA (p<0.05) $LSD_{0.05}$.

Fig. 5. Effect of different inocula in plants growing at three humidity conditions on nodulation and nitrogen accumulation.

We study the influence of three levels of water content on plants inoculated with different treatments: a conventional inoculum and two other previously induced with genistein. The experiment guaranteed severe and moderate drought stress condition in some plants (figure 4). The lowest values of Relative Water Content to 20 days to every treatment suggest that period when nodules were formed corresponded to the most critic phase of stress. The highest soil moisture level guaranteed better nodulation and a higher efficiency of this process were modulated by the inducer. (Williams & De Mallorca, 1984) demonstrated that the magnitude of stress effects and the rate of inhibition of symbiosis usually depended on the growth and development phase, as well as stress severity. In their results, mild water stress only reduced nodule number on soybean roots. Moderate and severe water stress reduced both nodule number and size. Our results showed a positive effect of genistein on nodulation, its efficiency and contribution to plant N nutrition at all soil moisture levels and was specially marked under the adverse conditions of drought stress (figure 5). Extensive research has focused on decreasing yield losses during soybean crop production. (Atti et al., 2005) found that foliar application of lipochitinoligosaccharides on soybean gave a positive effect on growth under moderate stress. Their results agree with ours, considering that they used LCO direct. In our work it was used as a *nod* gene inducer, which led to synthesis of Nod factors in the inocula. The effect of water lack on nodulation has been extensively documented (Franson et al., 1991; Sellstedt et al., 1993; Serraj et al., 1999). It is important to produce inoculants which have been obtained from induced media, because they will not only increase nodulation and N fixation, but can also help under adverse conditions of water stress. Other factors may be considered, such as plant growth stage. (Peña-Cabriales & Castellanos, 1993) found that water stress during vegetative growth was more detrimental to nodulation and N fixation than at the reproduction stage. In conclusion, after evaluating the effect of genistein as an inducer of *Bradyrhizobium japonicum* inoculants under water stress it was possible to show an important influence of this isoflavonoid on reducing the effect of water stress on nodulation (Nápoles, et al., 2009).

Many works have been conducting to understand the physiological mechanisms involved in soybean plants subjected to drought stress: leaf photosynthetic rates, carbohydrate concentrations, soluble invertase activities (Fulai et al., 2004), proline accumulation (Kolh et al., 1991; Curtis et al., 2004) among others. (Serraj et al., 1999) established that drought stress leads to a decrease of nitrogen fixation capacity, mainly as a consequence of ureides accumulation in shoots and asparagine in nodules. Several mechanisms have been reported to be involved in the physiological response, carbon shortage and nodule carbon metabolism, oxygen limitation, and feedback regulation by the accumulation of N fixation products, which results in poor nodulation and reduced amounts of fixed N (Zahran, 1999; Kurdalai et al., 2002; Serraj, 2003). Modifications in the activity of key nodule enzymes such as sucrose synthase and isocitrate dehydrogenase and in nodular malate content also occur. The decline in nodule water potential results in a cell redox imbalance (Marino et al., 2007). (Ladrera et al., 2007) demonstrated that drought reduced carbon flux and N accumulation in nodules, but not in shoots. Our group studied the ureides level in soybean treated with different inoculants in response to water deficit (Freixas et al., 2010). Soybean plants were firstly grown in nutrient solution for 20 days. Afterward, they were drought stressed for a 20 days period adding 10% polyethyleneglicol (PEG) 6000. *Bradyrhizobium elkanii* ICA 8001 was the strain used to inoculate soybean plants on this experiment, which was separately grown on three culture media, two of them induced in nodulation factor production. A statistically significant increase of ureides level in leaves and nodules was observed in plants with water

deficit and inoculants without nodulation factor induction (NFI). However, this increase was not observed in plants with water deficit and inoculants with NFI. These results suggest an important role of Nod factors also on ureides level regulation in soybean under drought conditions.

5.5 Inoculants and oligosaccharines

Oligosaccharines exert proven biological effects on the growth and development of plants and induce the expression of a variety of genes involved in defensive responses. The oligosaccharide part of the Nod factor structure from Rhizobium is also responsible for inducing cortical cell divisions of the root leading to form the nodular primordium. We study the influence of two oligosaccharines (partially hydrolyzed chitosan and a mixture of oligogalacturonides) on *Bradyrhizobium elkanii* ICA 8001 multiplication and on nodulation in soybean. The chitosan polymer was obtained by basic deacetylation of lobster chitin (Ramírez et al., 2000) and hydrolyzed for 24 h with Pectinex Ultra SP-L. The mixture of oligogalacturonides with a degree of polymerization between 7 and 16 residues of galacturonic acid was obtained from citrus pectin (Sigma), according to the methodology of (Cabrera, 2003). The partially hydrolyzed chitosan did not inhibit the *Bradyrhizobium* multiplication, whereas oligogalacturonic acid reduced the viability of the strain. The number of nodules developed showed the best results with the chitosan partially hydrolyzed at 10 and 100 mg L^{-1} (Costales et al., 2007). The positive effect of the hydrolyzed chitosan on the nodulation could be explained by several ways. It is known that the chitosan derivatives favor the plant growth and the radical system of several crops (Bitelli et al., 2001). As a result of these effects, the number and weight of the nodules could be benefit indirectly. On the other hand, the structure of nodulation factors contains basically a chitin oligosaccharide, which starts the process of nodule formation by inducing the cortical cell divisions on the roots leading to the formation of the nodular primordium. These signals mediate the entry of the microsymbiont and the process of nodulation (Macchiavelli & Brelles-Marino, 2004). Besides, foliar and seed application of chitosan oligosaccharides increase the isoflavonoids (genistein and daidzein) content in soybean seeds (Mal-Tawaha et al., 2005). These compounds constitute chemoatractans to *Bradyrhizobium* and specific inducers of *nod* genes activation in the bacteria. It would be valid the use of chitin derivatives as inoculant additives to increase the nodulation and soybean development. Further studies will be conducted to deepen into the structural requirements of oligosaccharines in the effect on symbiotic nodulation.

5.6 Inoculants as a product. Impact on agriculture

It is necessary to translate all of this knowledge in a product to be applied in big planting, to face the adverse condition, the competition, and then we can think our results contribute in a little way to perform a sustainable agriculture. Testing traditional and new induced inoculants on big extension of soybean, in different sites of Argentina, we can distinguish different results in yield. 78% of sites shown a positive response with the new product, with 243,12 $kg.ha^{-1}$ as average of yield´s increase and 188,07 $kg.ha^{-1}$ higher to the traditional inoculants (figure 6).

We evaluated the effect of some soil factors as pH, available phosphorus content, organic matter content, nitrates and *Bradyrhizobium* population, as well as the two inoculants on soybean yield (Nápoles, et al., 2009). The analysis of factors proved that, despite everyone

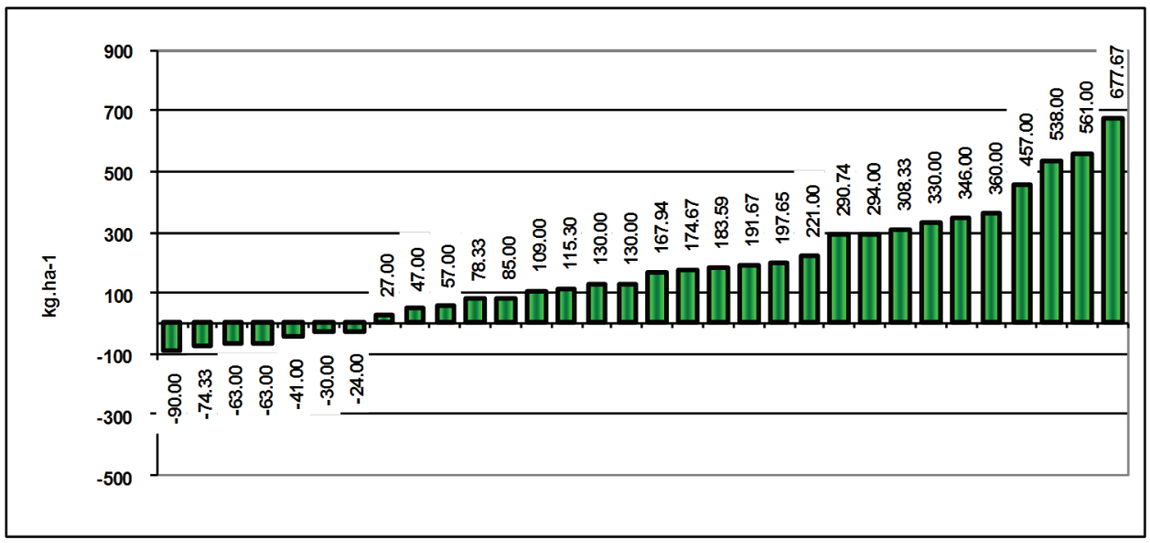


Fig. 6. Negative (Decrease) and Positive (Increase) effect of new inoculant on soybean yield in different sites.

influenced on it, only pH, *Bradyrhizobium* population existing in the soils tested and inoculant quality had a significant effect on yield. Plants growing on acid soils with a low bacteria population, which had been inoculated with the new induced biopreparation, showed higher yields. New products containing *Bradyrhizobium* as biological component in a good physiological state, increase competitiveness, assurance excellent nitrogen nutrition to the plant and guarantee higher yields.

6. Conclusions and future work

The outcome of the interaction *Bradyrhizobium*-soybean, as other legumes-rhizobium interaction, is dependent on an elaborate signal exchange that continues throughout the entire symbiotic process and has been likened to matching locks and keys (Broughton et al., 2000), with only the correct combination giving rise to efficient symbiosis. Taking into account that this symbiosis is in great measure responsible of nitrogen required by world agriculture (de Hoff & Hirsch, 2003), big efforts have done to improve this relation, specially related to the bacteria. It is very important that selective strains are effective, competitive. Our efforts have been focus on improve the physiological bacteria state to produce or activate the symbiosis determinants through culture media design, according to that purpose. We think that including natural sources of inducers it is possible not only activate the nodulation factor production, but also the protein secretion system and polysaccharides, all necessary to the symbiosis success. Nevertheless, we need to keep in mind all the factors related in that complex process, not only the bacteria, the plant, their genetic, physiology, also the soil and all the environmental factors biotic and abiotics, which can act on the system.

7. Acknowledgments

Authors want to thanks the National Institute of Agricultural Science in Cuba, the National Institute of Agronomical Technology in Argentina, the Centre of Microbial and Plant Genetics in Belgium, the McGill University in Canada and to Rizobacter Argentina S.A.

8. References

Abdel-Fattah, Y.; Saeed, H.; Gohar, Y. & Elbaz, M. (2005). Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. *Process Biochemistry*, 40, 5, 1707-1714.

An, J.; Carlson, R.; Glushka, J. & Streeter, J. (1995). The structure of a novel polysaccharide produced by *Bradyrhizobium* species within soybean nodules. *Carbohydr. Res.*, 269, 303–317.

Atti, S.; Bonnell, R.; Prasher, S. & Smith, D. (2005). Response of soybean {*Glycine max* (L.) Merr.} under chronic water deficit to LCO application during flowering and pod filling. *Irrigation and Drainage,* 54, 15-30.

Ausmees, N.; Kobayashi, H.; Deakin, W.; Marie, C.; Krishnan, H.; Broughton, W. & Perret, X. (2004). Characeterisation of NopP, a type III secreted effector of *Rhizobium* sp. NGR234. *J. Bacteriol*., 186, 4774–4780.

Bernal, G.; Illanes, A. & Ciampi, L. (2002). Isolation and partial purification of a metabolite from a mutant strain of *Bacillus sp*. *Electronic Journal of Biotechnology*, 5, 1.

Bhosale, P. & Gadre, R. (2001). Optimization of carotenoid production from hyper-producing *Rhodotorula glutinis* mutant 32 by a factorial approach. *Letters in Applied Microbiology,* 33, 12-16.

Bitelli, M.; Flury, M.; Campbell, G. & Nichols, E. (2001). Reduction of transpiration through foliar application of chitosan. *Agric. Forest Meteorol*., 107, 167.

Bosniols, A.; Chamalet, A.; Lagacherie, B.; Merrien, A. & Obaton, M. (1986). Le sojac physiologie de la plante et adaptation aux conditions francaises. Nutrition azotée du soja: limites et améliorations de la fixation symbiotique. *Cetiom,* 1867, 157-165.

Brencic, A. & Winans, S. (2005). Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiology and Molecular Biology Reviews*, 69, 155-194.

Broughton, W. J.; Jabbouri, S. & Perret. X. (2000). Keys to symbiotic harmony. *J. Bacteriol.,* 182, 5641–5652.

Broughton, W.J.; Hanin, M.; Relic, B.; Kopciñska, J.; Golinowski, W.; Simsek, S.; Ojanen-Reuhs, T.; Reuhs, B.; Marie, C.; Kobayashi, H.; Bordogna, B.; Le Quéré, A.; Jabbouri, S.; Fellay, R.; Perret, X & Deakin, W.J. (2006). Flavonoid inducible modifications to rhamnan O antigens are necessary for *Rhizobium* sp. strain NGR234-legume symbioses. *J. Bacteriol*., 188, 3654–3663.

Cabrera, J.C. (2003). Procedimiento de obtención de una mezcla de oligosacáridos pécticos estimuladora del enraizamiento vegetal. Cuban Patent, 22859.

Catroux, G.; Hartmann, A. & Revelim, C. (2001). Trends in rhizobial inoculant production and use. *Plant Soil,* 230, 21–30.

Costales, D.; Nápoles, M.C. & Falcón, A. (2007). Influence of chitosan and pectin oligosaccharides on the symbiotic interaction soybean-Bradyrhizobium. *Cuban Journal of Agricultural Science*, 41, 2, 167-173.

Curtis, J.; Shearer, G. & Kohl, D. (2004). Bacteroid Proline Catabolism Affects N_2 Fixation Rate of Drought-Stressed Soybeans. *Plant Physiology,* 136, 3313-3318.

D´Antuono, A.L.; Casabuono, A.; Couto, A.; Uglade, R.A. & Lepek, V.L. (2005). Nodule development induced by *Mesorhizobium loti* mutant strains affected in polysaccharide synthesis. *Molecular Plant-Microbe Interactions*, 18, 446-457.

D'Haeze, W. & Holsters, M. (2002). Nod factor structures, responses, and perception during initiation of nodule development. Glycobiology, *12, 6, 79R-105R.*

de Hoff, P. & Hirsch, A.M. (2003). Nitrogen comes down to earth: report from the 5th European Nitrogen Fixation Conference. *Mol. Plant Microbe Interact.*, 16, 371–375.

de Lyra, M.; do, C.C.P.; Lopez-Baena, F.J.; Madinabeitia, N.; Vinardell, J.M.; Espunyn, M.R.; Cubon, M.T.; Belloguin, R.A.; Ruiz-Sainz, J.E. & Ollero, F.J. (2006). Inactivation of the Sinorhizobium fredii *HH103 rhcJ gene abolishes nodulation outer proteins (Nops) secretion and decreases the symbiotic capacity with soybean.* Int. Microbiol.*, 9, 125–133.*

de Maagd, R.A.; Wijffelman, C.A.; Pees, E. & Lugtenberg, B.J. (1988). Detection and subcellular localization of two Sym plasmid dependent proteins of *Rhizobium leguminosarum* biovar *viciae*. *J. Bacteriol.*, 170, 4424–4427.

de Maagd, R.A.; Wijfjes, A.H.; Spaink, H.P.; Ruiz-Sainz, J.E.; Wijffelman, C.A.; Okker, R.J. & Lugtenberg, B.J. (1989). nodO, a new nod gene of the *Rhizobium leguminosarum* biovar *viciae* sym plasmid pRL1JI, encodes a secreted protein. *J. Bacteriol.*, 171, 6764–6770.

Deakin, W. J.; Marie, C.; Saad, M. M.; Krishnan, H. B. & Broughton, W. J. (2005). NopA is associated with cell surface appendages produced by the type III secretion system of *Rhizobium* sp. strain NGR234. *Mol. Plant-Microbe Interact.*, 18, 499–507.

Dusha, I.; Bakos, A.; Kondorosi, A.; de Bruijn, F.J. & Schell, J. (1989). The *Rhizobium meliloti* early nodulation genes (nodABC) are nitrogen-regulated: isolation of a mutant strain with efficient nodulation capacity on alfalfa in the presence of ammonium. *Mol. Gen. Genet.*, 219, 89-96.

Duzan, H.M.; Zhou, X.; Souleimanov A. & Smith, D.L. (2004). Perception of *Bradyrhizobium japonicum* Nod factor by soybean [*Glycine max* (L.) Merr.] root hairs under abiotic stress conditions. *Journal of Experimental Botany,* 55, 408, 2641-2646.

Economou, T. & Dalbey, R.E. (2004). Molecular cell research on protein export/secretion in bacteria. *Biochim. Biophys. Acta.*, 1694, 1–333.

Fauvart, M. & Michiels, J. (2008). Rhizobial secreted proteins as determinants of host specificity in the rhizobium-legume symbiosis. *FEMS Microbiol. Lett.*, 285, 1–9.

Franson, R.; Brown, M. & Bethlenfalvay, G. (1991). The *Glycine-Glomus-Bradyrhizobium* symbiosis. XI. Nodule gas exchange and efficiency as a function of soil and root water status in mycorrhizal soybean. *Physiol. Plant.,* 83, 476-482.

Frederick, J.; Camp, C. & Bauer, P. (2001). Drought-stress effects on branch and main stem seed yield and yield components of determinate soybean. *Crop Scienc*e, 41, 759-763.

Freixas, J.; Torres, W; Reynaldo, I.M. & Nápoles, M.C. (2010). Ureides level in soybean plants with different inoculants and water deficit. *Cultivos Tropicales*, in press.

Fulai, L.; Jensen, C. & Andersen, M. (2004). Drought stress effect on carbohydrate concentration in soybean leaves and pods during early reproductive development: its implication in altering podset. *Field Crops Research,* 86, 1-13.

Gage, D.J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews*, 68, 280-300.

Geurts, R.; Fedorova, E. & Bisseling, T. (2005). Nod factor signaling genes and their function in the early stages of *Rhizobium* infection. *Curr. Opin. Plant Biol.*, 346-352.

Ghosh, P. (2004). Process of protein transport by the type III secretion system. *Microbiol. Mol. Biol. Rev.* 68, 771–795.

Gómez, G. & Batista, C. (2006). Optimización de medios de cultivos para microorganismos, una valiosa estrategia para la producción de biopreparados de interés agrícola. *Cultivos Tropicales,* 27, 3, 17-24.

Graham, P.H. & Vance, C.P. (2000). Nitrogen fixation in perspective: an overview of research and extension needs. *Field Crop Res.*, 65, 93-106.

Hahn, M. & Hennecke, H. (1984). Localized mutagenesis in *Rhizobium japonicum*. *Mol. Gen. Genet*. 187, 419-425..

He, G.Q.; Kong, Q. & Ding, L.X. (2004). Response surface methodology for optimizing the fermentation medium of *Clostridium butyricum*. *Letters in Applied Microbiology,* 39, 4, 363-368.

Heidstra, R.; Yang, W.; Yalcin, Y.; Peck, S.; Emons, A.; Van Kammen, A. & Bisseling, T. (1997). Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in Rhizobium-legume interaction. *Development,* 124, 1781-1787.

Hubber, A.; Vergunst, A.C.; Sullivan, J.T.; Hooykaas, P.J. & Ronson, C.W. (2004). Symbiotic phenotypes and translocated effector proteins of the *Mesorhizobium loti* strain R7AVirB/D4 type IV secretion system. *Mol. Microbiol.* 54, 561–574.

Hume, D.J. & D.H. Blair. (1992). Effect of numbers of *Bradyrhizobium japonicum* applied in commercial inoculants on soybean seed yield in Ontario. *Can. J. Microbiol*. 38: 588–593.

Ip, H.; Aoust, F.D.; Begum, A.A.; Zhang, H.; Smith, D.L., Driscoll, B.T. & Charles, T.C. 2001. *Bradyrhizobium japonicum* mutants with enhanced sensitivity to genistein resulting in altered *nod* gene regulation. *Molecular Plant-Microbe Interactions.* 14, 12, 1404–1410.

Kaneko, T.; Nakamura, Y.; Sato, S. et al. (2000). Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res*., 7, 331–338.

Karr, D.B.; Liang, R.T.; Reuhs, B. & Emerich, D. (2000). Altered exopolysaccharides of *Bradyrhizobium japonicum* mutants correlate with impaired soybean lectin binding, but not with effective nodule formation. *Planta*, 211, 218-226.

Kim, H.O.; Lim, J.M.; Joo, J.H.; Kim, S.W.; Hwang, H.J.; Choi J.W. & Yun. J.W. (2005). Optimization of submerged culture condition for the production of mycelial biomass and exopolysaccharides by *Agrocybe cylindracea*. *Bioresource Technology,* 96, 10, 1175-1182.

King, C.A. & Purcell, L.C. (2001). Soybean nodule size and relationship to nitrogen fixation response to water deficit. *Crop Sci.*, 41, 1099–1107.

Kohl, D., Kennelly, E.; Zhu, Y.; Schubert, K. & Shearer G. (1991). Proline Accumulation, Nitrogenase (C_2H_2 reducing) Activity and Activities of Enzymes related to Proline Metabolism in Drought-Stressed Soybean Nodules. *Journal of Experimental Botany,* 42, 831-837.

Kosslak, R. M.; Bookland, R.; Barkei, J.; Paaren, H.; & Appelbaum, E. R. (1987). Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. *Proc. Natl. Acad. Sci. USA,* 84, 7428-7432.

Krause, A.; Doerfel, A. & Gottfert, M. (2002). Mutational and transcriptional analysis of the type III secretion system of *Bradyrhizobium japonicum*. *Mol. Plant–Microbe Interact.*, 15, 1228–1235.

Krishnan, H.B.; Lorio, J.; Kim, W.S.; Jiang, G.; Kim, K.Y.; De Boer, M. & Pueppke, S.G. (2003). Extracellular proteins involved in soybean cultivar-specific nodulation are associated with pilus-like surface appendages and exported by a type III protein secretion system in *Sinorhizobium fredii* USDA257. *Mol. Plant–Microbe Interact.*, 16, 617–625.

Kurdalai, F.; Al-ain, F. & Al-shamm A, M. (2002). Nodulation, dry matter production, and N2 fixation by faba bean and chickpea as affected by soil moisture and potassium fertilizer. *J. Plant Nutr.,* 25, 355-368.

Ladrera, R.; Marino, D.; Larrainzar, S.; González, E. & Arrese-Igor, C. (2007). Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. *Plant Physiol*., 145, 539-546.

Laeremans, T.; Coolsaet, N.; Verreth, C.; Snoeck, C.; Hellings, N.; Vanderleyden, J. & Martínez-Romero, E. (1998). Functional redundancy of genes for sulphate activation enzymes in *Rhizobium* sp. BR 816. *Microbiology*, 143: 3933-3942.

Lee, K.M. & Gilmore, D.F. (2005). Formulation and process modelling of biopolymer (polyhydroxyalkanoates: PHAs) production from industrial wastes by novel crossed experimental design. *Process Biochemistry,* 40, 1, 229-246.

Lee, T.M.; Chen W.C. & Chou. C.C. (1998). Maximization of cholesterol oxidase production by *Rhodococcusequi* no. 23 by using response surface methodology. *Biotechnol. Appl. Biochem*., 28, 229–233.

Lepek, V. & D´Antuono, A.L. (2005). Bacterial surface polysaccharides and their role in the rhizobia-legume association. *Lotus Newsletter*, 35, 1, 93-105.

Lerouge, I. & Vanderleyden, J. (2001). O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. *FEEMS Microbiology Reviews*, 26, 17-47.

Lhuissier, F.G.P.; De Ruijter, N.C.A.; Sieberer, B.J.; Esseling, J.J. & Emons, A.M.C. (2001). Time course of cell biological events evoked in legume root hairs by *Rhizobium* Nod factors: state of the art. *Ann. Bot.*, 87, 289–302.

Loh, J. & Stacey, G. (2003). Nodulation gene regulation in *Bradyrhizobium japonicum*: a unique integration of global regulatory circuits. *Appl. Environ. Biol*., 69, 10–17.

Long, S. R. (1996). *Rhizobium* symbiosis: Nod factors in perspective. *Plant Cell,* 8, 1885-1898.

López, M. (1990). Manual de Tecnologías de Producción del biofertilizante Bio-Rhizo. La Habana, Cuba.

Lupwayi, N.Z., Olsena, P.E.; Sandeb, E.S.; Keyserb, H.H.; Collinsa, M.M.; Singleton P.W. & Rice. W.A. (2000). Inoculant quality and its evaluation. *Field Crops Res*., 65, 259–270.

Macchiavelli, R.E. & Brelles, G. (2004). Nod factor-treated Medicago truncatula roots and seeds show an increased number of nodules whit inoculated with a limiting population of *Sinorhizobium meliloti*. *J. Exp. Bot.* 55, 26-35.

Mal-Tawaha, A.; Seguin, P.; Smith, D.L. & Beaulieu, C. (2005). Biotic elicitors as of increasing isoflavone concentration of soybean seeds. *Ann. Appl. Biol.* 146, 303.

Marie, C.; Deakin, W. J.; Viprey, V.; Kopcinska, J.; Golinowski, W.; Krishnan, H. B.; Perret, X. & Broughton, W. J. (2003). Characterisation of Nops, nodulation outer proteins, secreted via the type III secretion system of NGR234. *Mol. Plant-Microbe Interact*. 16, 743–751.

Marie, C.; Deakin, W.J.; Ojanen-Reuhs, T; Diallo, E; Reuhs, B; Broughton, W.J. & Perret, X. (2004). TtsI, a key regulator of Rhizobium species NGR234 is required for type III-dependent protein secretion and synthesis of rhamnose-rich polysaccharides. *Mol. Plant–Microbe Interact.*, 17, 958–966.

Marino, D.; Pierre, F.; Ladrera, R.; Zabalza, A.; Puppo, A.; Arrese-Igor, C. & González, E. (2007). Nitrogen fixation control under drought stress. Localized or systemic? *Plant Physiol.* 143, 1968-1974.

Martinez-Abarca, F.; Herrera-Cervera, J.A.; Bueno, P.; Sanjuan. J.; Bisseling. T. & Olivares, J. (1998). Involvement of salicylic acid in the establishment of the *Rhizobium meliloti*–alfalfa symbiosis. *Mol. Plant Microbe Interact.*, 11, 153–155.

Michiels, J.; Dombrecht, B.; Vermeiren, N.; Xi, C.W.; Luyten, E. & Vanderleyden, J. (1998). *Phaseolus vulgaris* is a non-selective host for nodulation. *FEMS Microbiology Ecology,* 26, 193-205.

Miransar, M. & Smith, D. (2009). Rhizobial Lipo-Chitooligosaccharides and Gibberellins Enhance Barley (Hordeum vulgare L.) Seed Germination. *Biotechnology,* 8, 2, 270-275.

Nápoles, M. C.; Guevara, E.; Montero, F.; Rossi, A. & Ferreira, A. (2009). Role of *Bradyrhizobium japonicum* induced by genistein on soybean stressed by water deficit. *Spanish Journal of Agricultural Research*, 7, 3, 665-671.

Nápoles, M.C. (2002). Medio de cultivo para *Bradyrhizobium japonicum*. Biopreparado resultante. Cuban Patent, 22797.

Nápoles, M.C.; González-Anta, G.; Cabrera, J.C.; Varela, M.; Guevara, E.; Meira, S.; Nogueras, F. & Cricco, J. (2009). Influencia de inoculantes y factores edáficos en el rendimiento de la soya. *Cultivos Tropicales*, 30, 3, 18-22.

Nápoles, M.C.; Luyten, E.; Dombrecht, B. & Vanderleyden, J. (2003). *Bradyrhizobium elkanii* ICA 8001 *gus* A: a new strain to evaluate nodulation gene expression. *Cultivos Tropicales*, 24, 3, 33-37.

Nápoles, M.C.; Luyten, E.; Dombrecht, B.; Laeremans, T.; Vanderleyden, J.; Costales, D.; Gutiérrez, A. & Corbera, J. (2005). Growth media modulating the Symbiotic Efficiency of *Bradyrhizobium elkanii*. *Symbiosis*, 38, 1, 87-98.

Nápoles, M.C.; Martínez, J.; Costales, D.; Gómez, G. & Somers, E. (2006). Efecto de diferentes medios de cultivo en la multiplicación celular de *Bradyrhizobium elkanii*. *Cultivos Tropicales*, 27, 1, 35-38.

Nawani, N.N. & Kapadnis, B.P. (2005). Optimization of chitinase production using statistics based experimental designs. *Process Biochemistry,* 40, 2, 651-660.

Noel, K.D.; Forsberg, L.S. & Carlson, R.W. (2000). Varying the abundance of O antigen in *Rhizobium etli* and its effect on symbiosis with *Phaseolus vulgaris*. *Journal of Bacteriology*, 182, 5317-5324.

Ohashi, Y.; Saneoka, H. & Fujita, K. 2000. Effect of water stress on growth, photosynthesis, and photoassimilate translocation in soybean and tropical pasture legume siratro. *Soil Sci. Plant Nutr.*, 46, 417-425.

Pan, B.; Zhang, F. & Smith, D.L. (1998). Genistein addition to the soybean rooting medium increases nodulation. *Journal of Plant Nutrition,* 21, 1631–1639.

Papanikou, E.; Karamanou, S. & Economou, A. (2007). Bacterial protein secretion through the translocase nanomachine. *Nat. Rev. Microbiol.,* 5, 839–851.

Parekh, S.; Vinci, V.A. & Strobel, R.J. (2000). Improvement of microbial strains and fermentation processes. *Applied Microbiology and Biotechnology,* 54, 287-301.

Parniske, M.; Schmidt, P.; Kosch, K. & Mller, P. (1994). Plant defense responses of host plants with determinate nodules induced by EPS-defective *exoB* mutants of *Bradyrhizobium japonicum*. *Mol. Plant-Microbe Interact.,* 5, 631–638.

Pellock, B.J.; Cheng, H. & Walker G.C. (2000). Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. *Journal of Bacteriology*, 182, 4310-4318.

Pena-Cabriales, J. & Castellanos, J. (1993). Effect of water stress on N_2 fixation and grain yield of *Phaseolus vulgaris* L. *Plant Soil.*, 152, 151-155.

Perret, X.; Staehelin, C. & Broughton, W.J. (2000). Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.*, 64, 180–201.

Pijeira, L. & Treto, E. (1983). Estudio del comportamiento de las cepas de *Rhizobium japonicum* asociadas a variedades de soya de primavera. *Cultivos Tropicales,* 5, 1, 61-73.

Pijeira, L.; Treto, E.; Mederos, J.D.; Corbera, J.; Velazco, A.; Castellanos M. & Medina, N. (1988). La nutrición y fertilización de la soya cultivada en condiciones de un suelo ferralítico rojo compactado de Cuba. *Cultivos Tropicales,* 10, 3, 19-26.

Prithiviraj, B.; Souleimanov, A.; Zhou, X. & Smith, D.L. (2000). Differential response of soybean (Glycine max (L.) Merr.) to lipochitooligosaccharide Nod Bj V(C18:1 Me Fuc). *J. Exp. Bot.*, 51, 2045–2051.

Prithiviraj, B.; Zhou, X.; Souleimanov, A.; Kahn, W.M. & Smith, D.L. (2003). A host-specific bacteria-to-plant signal molecule (Nod factor) enhances germination and early growth of diverse crop plants. *Planta*, 216: 437–445.

Purcell L., Serraj R., Sinclair T. & De A. 2004. Soybean N_2 Fixation Estimates, Ureide Concentration, and Yield Responses to Drought. *Crop Science*, 44:484-492.

Ramírez, M.; Cabrera, G.; Gutiérrez, A. & Rodríguez, T. (2000). Metodología para la obtención de quitosana a bajas temperaturas. *Cult. Trop.* 21, 79.

Relic, B.; Perret, X.; Estradarcía, M.T.; Kopcinsca, J. & Golinowski, W. (1994). Nod factors of *Rhizobium* are the key to the legume door. *Mol. Microbiol*. 13, 171-78.

Richardson, A.E.; Djordjevic, M.A.; Rolfe, B.G. & Simpson R.J. (1988). Effects of pH, Ca and Al on the exudation from clover seedlings of compounds that induce the expression of nodulation genes in *Rhizobium trifolii*. *Plant and Soil.*, 109, 37-47.

Robson, A.D. & Bottomley, P. J. (1991). Limitations in the use of legumes in agriculture and forestry. In *The Biology and Biochemestry of Nitrogen Fixation,* M.J. Dilworth & A.R. Glenn (Eds.), 320-249. Elsevier, Amsterdam.

Saad, M.M.; Kobayashi, H.; Marie, C.; Brown, I.; Mansfield, J.W.; Broughton, W.J. & Deakin, W.J. (2005). NopB, a type III secreted protein of *Rhizobium* sp. strain NGR234, is associated with pilus-like surface appendages. *J. Bacteriol.,* 187, 1173–1181.

Schlaman, H.R.; Gisel, A.A.; Quaedvlieg, N.E.; Bloemberg, G.V.; Lugtenberg, B.J.; Kijne, J.W.; Potrykus, I.; Spaink, H.P. & Sautter, C. (1997). Chitin oligosaccharides can induce cortical cell division in roots of Vicia sativa when delivered by ballistic microtargeting. *Development*, 124, 4887–4895.

Schultze, M. & Kondorosi, A. (1998). Regulation of symbiotic root nodule development. *Annual Review of Genetics,* 32, 33-57.

Schultze, M.; Kondorosi, E.; Ratet, P.; Buir´e, M. & Kondorosi, A. (1994). Cell and molecular biology of *Rhizobium*-plant interactions. *Int. Rev. Cytol.,* 156, 1–75.

Sellstedt, A.; Staahl, L.; Mattsson, M.; Jonsson, K. & Hoegberg, P. (1993). Can the ^{15}N dilution technique be used to study N_2 fixation in tropical tree symbioses as affected by water deficit?. *J. Exp. Bot.*, 44, 1749-1755.

Senthilkumar, S.R.; Ashokkumar, Chandra, B.K.R. & Gunasekaran, P. (2005). Optimization of medium composition for alkali-stable xylanase production by *Aspergillus fischeri* Fxn 1 in solid-state fermentation using central composite rotary design. *Bioresource Technology,* 96, 12, 1380-1386.

Serraj, R. (2003). Effects of drought stress on legume symbiotic nitrogen fixation: Physiological mechanisms. *Indian J. Exp. Biol*. 41, 1136-1141.

Serraj, R.; Sinclair, T. & Purcell, L. (1999). Symbiotic N2 fixation response to drought. *J. Exp. Bot.*, 50, 143-155.

Singleton, P.W.; Boonkerd, N.; Carr, T.J. & Thompson J.A. (1997). Technical and market constraints limiting legume inoculant use in Asia. *Proceedings of an International*

Workshop on Managing Legume Nitrogen Fixation in the Cropping Systems of Asia. Pp. 17-38, ICRISAT Asia Centre, Andhra Pradesh, India.

Smith, D.L. & Zhang, F. (1999). Composition for enhancing grain yield and protein yield of legumes grown under environmental condition that inhibit or delay nodulation thereof. US Patent, 5922316.

Spaink, H.P. (2000). Root nodulation and infection factors produced by rhizobial bacteria. *Annu. Rev. Microbiol*., 54, 257-288.

Stokkermans, T. & Peters N. (1994). *Bradyrhizobium elkanii* lipo-oligosaccharide signals induce complete nodule structures on *Glycine soja*. *Planta*, 193, 413-420.

Streeter, J.G. (1988). Inhibition of legume nodule formation and N_2 fixation by nitrate. *Crit. Rev. in Plant Sci*., 7, 1-23.

Streeter, J.G. (2003). Effects of drought on nitrogen fixation in soybean root nodules. *Plant Cell Environ*., 26, 1199- 1204.

Supanjani, S.; Lee, K.D.; Almaraz, J.J.; Zhou, X. & Smith. D.L. (2006). Effect of organic N source on bacterial growth, lipo-chitooligosaccharide production, and early soybean nodulation by *Bradyrhizobium japonicum*. *Can. J. Microbiol*., 52, 227–236.

Tajima, S.; Nomura, M. & Kouchi, H. (2004). Ureide biosynthesis in legume nodules. *Front Biosci*., *9*, 1374–1381.

Treto, E.; García, M.; Martínez, R. & Febles, J.M. (2005). Avances en el manejo de los suelos y la nutrición orgánica. Available on: http://www.laneta.apc.org/desal/spip/article.php3?id_article=29

Vijn, I.; Das Neves, L.; Kammena, V; Franssen, H. & Bisseling, T., (1993). Nod factors and nodulation in plants. *Science*, 260, 1764-1765.

Vincent, J.M. (1970). A manual for the practical study of root-nodule bacteria. In *International Biological Programme Handbook No. 15*, Blackwele scientific publications, Oxford, England.

Viprey, V.; Del Greco, A.; Golinowski, W.; Broughton, W.J. & Perret, X. 1998. Symbiotic implications of type III protein secretion machinery in *Rhizobium*. *Mol. Microbiol*., 28, 1381–1389.

Wang, Y.X. & Lu, Z. (2005). Optimization of processing parameters for the mycelial growth and extracellular polysaccharide production by *Boletus* spp. ACCC 50328. *Process Biochemistry,* 40, 3-4, 1043-1051.

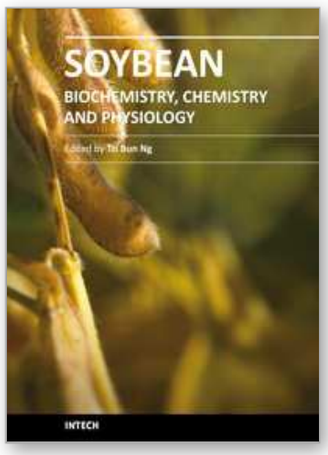
Williams, P. & De Mallorca, M. (1984). Effect of osmotically induced leaf moisture stress on nodulation and nitrogenase activity of *Glycine max*. *Plant Soil,* 80, 267-283.

Zahran, H. (1999). Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews,* 63, 968-989.

Zhang, F. & Smith, D.L. (1996). Inoculation of soybean (*Glycine max*. (L.) Merr.) with genistein-preincubated *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season condition, *Plant Soil*, 179, 233–241.

Zhang, F.; Dijak, M.; Smith, D.L.; Lin, J.; Walsh, K.; Voldeng, H.; Macdowell, F. & Layzell, C.D. (1997). Nitrogen fixation and nitrate metabolism for growth of six diverse soybean (*Glycine max* (L) Merr.) genotypes under low temperature stress. *Environ. Exp. Bot*. 38, 49-60.

Zhang, J.; Zhang, X. & Liang, J. (1995). Exudation rate and hydraulic conductivity of maize roots are enhanced by soil drying and abscisic acid treatment. *New Phytologist*, 131, 329-336.



Soybean - Biochemistry, Chemistry and Physiology
Edited by Prof. Tzi-Bun Ng

ISBN 978-953-307-219-7
Hard cover, 642 pages
Publisher InTech
Published online 26, April, 2011
Published in print edition April, 2011

Soybean is an agricultural crop of tremendous economic importance. Soybean and food items derived from it form dietary components of numerous people, especially those living in the Orient. The health benefits of soybean have attracted the attention of nutritionists as well as common people.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Nápoles Marîa C, Gómez Gretel, Costales Daimy, Freixas JA, Guevara E, Meira S, González-Anta G and Ferreira A (2011). Signals in Soybean's Inoculants, Soybean - Biochemistry, Chemistry and Physiology, Prof. Tzi-Bun Ng (Ed.), ISBN: 978-953-307-219-7, InTech, Available from: http://www.intechopen.com/books/soybean-biochemistry-chemistry-and-physiology/signals-in-soybean-s-inoculants

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen