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Review article

Soybean interactions with soil microbes, agronomical and molecular aspects

D.N. Rodríguez-Navarro[1]*, I. Margaret Oliver[2], M. Albareda Contreras[1], J.E. Ruiz-Sainz[2]

[1] IFAPA, Centro Las Torres-Tomejil. Apartado Oficial 41200, Alcalá del Rio, Seville, Spain
[2] Department of Microbiology, Faculty of Biology, University of Seville, Seville, Spain

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Abstract – Soybean, *Glycine max* (L.) Merrill, is one of the most important food crops in the world. High soybean yields require large amounts of N fertilizers, which are expensive and can cause environmental problems. The industrial fixation of nitrogen accounts for about 50% of fossil fuel usage in agriculture. In contrast, biological fixation of N_2 is a low-cost source of N for soybean cropping through the symbiotic association between the plant and soil bacteria belonging to the genera *Bradyrhizobium* and *Sinorhizobium*, which are collectively called "soybean rhizobia". In general, symbiotic nitrogen fixation in crop legumes not only reduces fertilizer costs but also improves soil fertility through crop rotation and intercropping. Biological nitrogen fixation is due to symbioses between leguminous plants and species of *Rhizobium* bacteria. Replacing this natural N source by synthetic N fertilizers would cost around 10 billion dollars annually. Moreover, legume seed and foliage have a higher protein content than that of non-legumes, and this makes them desirable protein crops. There is a wide knowledge of the industrial elaboration and use of commercial soybean inoculants based on bradyrhizobia strains. At present, the technology to prepare different types of inoculants, either solid or liquid, is sufficiently developed to meet market requirements, although further research and investments are still required to improve the symbiotic efficacy of rhizobial inoculants. Inoculation of soybeans under field conditions has been successful in the USA, Brazil and Argentina, which are the world leaders in soybean cultivation in terms of acreage and grain yields. There are, however, limitations to a wider use of rhizobial inoculants: the size of indigenous soil rhizobial populations can prevent the successful use of inoculants in some particular areas. For example, many Chinese soils contain more than 10^5 soybean rhizobia per gram of soil, which imposes a serious barrier for nodule occupancy by the soybean rhizobia used as an inoculant. The use of inoculants based on soil bacteria other than rhizobia has also increased in the last decades. An example is the genus *Azospirillum,* which can be used for its capacity to increase plant growth and seed yields through different mechanisms, such as the production of plant hormones and the increase in phosphate uptake by roots. In addition, co-inoculation with *Azospirillum* and rhizobia enhances nodulation and nitrogen fixation. Although less developed, it is expected that inoculants based on mycorrhizal fungi will also play a relevant role in sustainable agriculture and forestry. In spite of any possible limitations, the use of inoculants appears compulsory in a frame of sustainable agriculture, which seeks to increase crop yields and nutrient-use efficiency while reducing the environmental costs associated with agriculture intensification. This review also summarizes some of the most relevant genetic aspects of soybean rhizobia in relation to their symbiosis with soybeans. They can be listed as follows: (1) legume roots exude flavonoids, which are able to activate the transcription of nodulation (*nod*, *nol*, *noe*) genes; (2) expression of nodulation genes results in the production and secretion of lipo-chitin oligosaccharide signal molecules, called LCOs or "Nod factors", which activate nodule organogenesis in the legume root; (3) LCOs induce numerous responses of the legume roots, such as hair curling and the formation of nodule primordia in the inner or outer cortex; (4) the function of many soybean rhizobia *nod* genes is known and the chemical structure of the LCOs produced has been determined; (5) in addition to LCOs, different soybean rhizobia surface polysaccharides are required for the formation of nitrogen-fixing nodules; (6) surface polysaccharides might act as signal molecules or could prevent plant defense reactions. Cyclic glucans, capsular polysaccharides and lipopolysaccharides appear to play relevant roles in the soybean nodulation process since rhizobial mutants affected in any of these surface polysaccharides are symbiotically impaired. Present knowledge of the molecular bases determining cultivar-strain specificity and nodule occupancy by soybean rhizobia competitors is clearly insufficient. This lack of information is a serious barrier for developing strategies aimed at improving nodulation and symbiotic nitrogen fixation of commercial inoculants. In spite of these difficulties, recent studies have shown that the signaling pathway involved in triggering nodule organogenesis is independent of that operating in bacterial entry through infection thread formation. Theses facts might offer new insights for improving symbiotic nitrogen fixation and also for the feasibility of transferring nodule organogenesis, a first step in expanding this symbiotic interaction into other agriculturally important species.

soybean / legume inoculants / *Bradyrhizobium japonicum* / *Sinorhizobium fredii* / nodulation factors / rhizobial surface polysaccharides

* Corresponding author: dulcenombre.rodriguez@juntadeandalucia.es

Contents

1. INTRODUCTION

1.1. Brief history of world soybean production

The soybean [*Glycine max* (L.) Merrill] is one of the most important food crops in the world. High soybean yields require large amounts of nitrogen (N). The least expensive source of N for soybean is biological fixation of atmospheric N_2 by the symbiotic association between the plant and soil bacteria belonging mainly to the genus *Bradyrhizobium*. The efficiency of the biological fixation of the atmospheric N_2 process depends on many factors related to the plant, to the bacteria, to the symbiosis, and to the environment. Problems of low soil fertility and limited availability of macro- and micronutrients are also among the most important constraints (Campo et al., 2009).

By whom, when, where and how the soybean crop was domesticated in China and disseminated throughout the world is a fascinating story (Hymowitz and Shurtleff, 2005). Because the soybean is one of humanity's food crops, the history and literature describing the origin of soybean cultivation is full of errors and myths. In fact, soybean is not one of the world's oldest domesticated crops and there are no archaeological records that suggest the soybean has been cultivated in China for more than 5000 yr. The current oldest reliable record for soybean in China goes back to the 11th century BC or perhaps a little earlier.

The earliest known introduction of the soybean into America was by Samuel Bowen in 1765. He introduced Chinese vetches (soybean) into the Colony of Georgia. Henry Yonge, the Surveyor General of Georgia from 1766 on, cultivated soybean at the request of Bowen and one year later he received a royal patent for making soy sauce from soybean growing in America. The term "soybean" was probably coined to refer to the bean from which soy sauce was produced.

In 1712 the German botanist Kaempfer brought soybean to Europe. Soybean was exhibited in the Botanical Garden of Paris in 1739 and in the British Royal Botanic Gardens in 1790. Soybean cropping was initiated in other countries in Europe (1875–1877) as a result of its successful exhibition at an International Exposition in Vienna in 1873. Although it was not cropped in Brazil until the 1960s, at present Brazil is the second largest soybean producer after the United States of America. Soybean cropping was spread through Africa during the twentieth century.

1.2. The importance of soybean cultivation today

The US soybean harvest reached over 10 million tons (Mt) in the early 1950s and it is now 70–75 Mt a year, almost half of the global total. Soybeans are the country's second most valuable crop, close behind corn, and worth nearly three times as much as wheat. Soybean acreage represents more than 15% of all arable land. Brazilian production, stimulated by exports, has grown even faster: the area planted with soybeans has expanded more than sixty-fold since the late 1950s and the total national production in 2007 reached 61 Mt. In the same year, Argentina with acreage of about 18 Mha harvested 47 Mt, relegating the Chinese harvest to fourth place. The Japanese now grow a mere 3% of what they consume.

In Europe acreage dedicated to soybean is less than 1Mha (ca. 840 000 ha). Italy (with 44.5%), France (29%) and Romania (22%) occupy the leading positions. Grain yields vary between 3300 and 2500 kg/ha in Italy and France, respectively. Table I summarizes the total acreage, production and value of soybean, corn and wheat crops of the United States of America during the period 2006–2008. Table II shows the production of the leading soybean countries as well as the main sources of protein meal consumption. Interestingly, although many fishes are close to extinction due to overfishing, fish protein meal consumption is only 3% of that derived from soybean.

This review is mainly focussed on the description of applied and fundamental aspects of rhizobia species able to form nitrogen-fixing nodules on soybean roots. Other soil microorganisms that, through their interaction with soybean roots, show a positive effect on plant growth will be briefly mentioned. Information about the plant side can be found at the website "http://www.wsrc2009.cn/en/index.asp", which includes The Proceedings of the VIII World Soybean Research

Table I. Harvested acreage, total production, and value of soybean, corn and wheat in the 2006–2008 cropping seasons in the USA.

Crop	Year	Harvested acreage (Mha)	Production (Mt)	Value of production (billion dollars)
Soybean	2008	30.2	79	27.3
	2007	25.9	72	26.9
	2006	30.2	86	20.4
Corn	2008	31.8	302	47.4
	2007	35.0	326	54.7
	2006	28.6	263	32.1
Wheat	2008	22.5	67	16.6
	2007	20.6	55	13.3
	2006	18.9	49	7.7

Legend: Mha, million hectares; Mt, million tons.
Source: NASS, National Agriculture Statistics Service of the United States Department of Agriculture (USDA).

Table II. World soybean production and main sources of protein meal consumption in 2007.

World Soybean Production (Million Tons)						
Country	USA	Brazil	Argentina	China	India	Paraguay
	70.4	61.0	47.0	14.3	9.3	7.0
World Protein Meal Consumption (Million Tons)						
Source	Soybean	Rapeseed	Cottonseed	Sunflower	Peanut	Fish
	160.2	27.1	15.3	10.6	5.9	5.3

Legend: Data presented in this table are from "Source, Soy Stats® of the American Soybean Association".

Conference (Peking, 2009). Updated reports about many applied and fundamental aspects related to soybeans (such as "soybean industry", "soybean germplasm collections", "genetics and breeding", or "farming system and management") can easily be found at this web address.

2. SOIL MICROORGANISMS INTERACTING WITH SOYBEAN

Microorganisms showing beneficial effects on soybean growth can be classified into two main groups: biofertilizers and the so-called Plant Growth-Promoting Rhizobacteria (PGPR). The term biofertilizer can be defined as a product which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. This definition is based on the logic that the term biofertilizer is a contraction of the term *biological fertilizer*.

Bacteria belonging to the 'rhizobia' group, the arbuscular mycorrhizal fungi and *Azospirillum* sp. are the main microorganisms used as biofertilizers in soybean agronomic practices. Members of the rhizobia group supply nitrogen through the establishment of the symbiotic rhizobia-legume association, while arbuscular mycorrhizal fungi supply mineral nutrients, mainly phosphorus and water. The positive effects of *Azospirillum* on plant growth are not mainly due to biological nitrogen fixation but to the bacterial capacity to produce phytohormones. These bacterial substances, which induce morphological and physiological changes in the inoculated plant root, would lead to an enhancement of water and mineral uptake.

The rhizobia group comprises microorganisms that belong to different bacterial genera, all of which are Gram-negative and heterotrophic. They are aerobic bacteria, although growth under conditions of low oxygen concentration can also take place. Their optimal growth conditions are at pH 7 and 28–30 °C. Traditionally, two main groups of rhizobia have been distinguished:

– fast-growing rhizobial strains with a generation time of about 1.5–4.0 h in yeast mannitol medium. Many of these strains belong to the genera *Rhizobium*, *Sinorhizobium* or *Mesorhizobium*. These strains usually acidify media containing mannitol, such as yeast mannitol, or other hexoses as the carbon source;
– slow-growing rhizobial strains with a generation time of at least 6 h, many of them belonging to the genus *Bradyrhizobium*. These strains usually alkalinize yeast mannitol medium.

Although this characteristic based on the speed of growth in laboratory media might seem very artificial, it actually reflects deep phylogenetic divergences. In other words, fast-growing rhizobia are clearly different from slow-growing ones. In addition, neither the fast-growing nor the slow-growing groups are homogeneous, since intragroup divergences are also significant.

Soybeans are nodulated by fast- and slow-growing rhizobia, which are collectively called "soybean rhizobia". Since soybean has been cropped in China for many centuries, it is reasonable to expect that Chinese soils are very rich in a high diversity of rhizobia able to nodulate this legume. This idea is based on the hypothesis that co-evolution between the two symbionts, the plant and the bacteria, has taken place over a long period of time, and, consequently, it should be possible to isolate in China different, or even new, soybean rhizobia species and also bacterial strains showing superior symbiotic performance with soybeans. Taxonomic studies carried out in the last 30 years have proved that this assumption is true, since new soybean rhizobia able to nodulate soybeans have been isolated from many distinct geographical areas of China.

Until 1982, soybeans were believed to be nodulated only by slow-growing bacteria belonging to the species

Table III. Different genera and species of soybean rhizobia.

Genus	Species	Speed of growth in YM medium	References
Bradyrhizobium	*japonicum*	slow	Jordan (1982)
	elkanii	slow	Kuykendall et al. (1992)
	liaoningense	slow	Xu et al. (1995)
Mesorhizobium	*tianshanense*	slow	Chen et al. (1995)
Sinorhizobium	*fredii*	fast	Chen et al. (1988)
	xinjiangense	fast	Chen et al. (1988)

YM, Yeast Mannitol.

Bradyrhizobium japonicum. Since then, however, new groups of soybean rhizobia have been isolated and classified. Table III lists the different genera and species comprising soybean-nodulating rhizobia.

All slow-growing soybean rhizobia were originally grouped in the species *B. japonicum*. However, serological and DNA homology divergences among the slow-growing soybean rhizobia, as well as differences in multilocus enzyme electrophoresis analyses and variations in antibiotic resistance patterns, exopolysaccharides, fatty acids and hemoproteins led to the segregation of a subset of strains in the new species *B. elkanii* (Kuykendall et al.,1992).

Strains belonging to *B. liaoningense* were isolated from different Chinese provinces such as Liaoning, Heilongjiang and Hubei (Xu et al., 1995). The doubling time of these bacteria is so long (it ranges from 19 to 39 h) that they are commonly referred to as extra slow-growing soybean rhizobia. Some strains are highly effective with some Asiatic soybeans, such as the cultivar Heinong 26.

Mesorhizobium tianshanense strains, originally called *Rhizobium tianshanense*, were isolated from an arid saline desert soil in the Chinese Xinjiang province (Chen et al., 1995). The strains comprising the species *M. tianshanense* were isolated from nodules of different legumes, such as *Glycyrrhiza padilliflora*, *G. uralensis*, *Sophora alopecuroides* and *Glycine max*. *M. tianshanense* strains produce acid in medium containing mannitol and their generation times vary between 5 and 15 h.

Fast-growing soybean bacteria were first isolated in the early 1980s (Keyser et al., 1982) and were finally clustered into the species *Sinorhizobium fredii* (Chen et al., 1988). These first soybean-nodule isolates are able to form nitrogen-fixing nodules on Asiatic soybean cultivars (such as the cultivar Peking) but fail to nodulate, or are very poorly effective, with the modern cultivars from North America (Keyser et al., 1982; Buendía-Clavería and Ruiz-Sainz, 1985; Buendía-Clavería et al., 1989). Subsequently, the isolation of new *S. fredii* strains that were able to form nitrogen-fixing nodules with both Asiatic and American soybean cultivars was reported (Dowdle and Bohlool, 1985; Yang et al., 2001; Videira et al., 2001; Camacho et al., 2002; Thomas-Oates et al., 2003). In contrast with the marked soybean cultivar specificity of some *S. fredii* strains, *S. fredii* strains show a very broad host range, being able to nodulate at least 79 different genera of legumes (Pueppke and Broughton, 1999).

Most of the well-studied strains of *S. fredii* are from China (Keyser et al., 1982; Dowdle and Bohlool, 1985; Yang et al., 2001; Camacho et al., 2002; Thomas-Oates et al., 2003), although fast-growing soybean microsymbionts have also been isolated from other geographical areas, such as Malaysia (Young et al., 1988), Vietnam (Rodríguez-Navarro et al., 1996) and Panama (Hernández and Focht, 1984).

Taxonomic studies have shown that *S. fredii* is closely related to the alfalfa microsymbiont *Sinorhizobium meliloti*. They are also related to *Sinorhizobium teranga* and *S. saheli* (de Lajudie et al., 1994), two species isolated from nodules of West African trees. The list of species belonging to the genus *Sinorhizobium* has been enlarged in the last 15 years and it covers rhizobial strains from Africa and America that nodulate a vast variety of legumes, such as *Acacia*, *Prosopis*, *Kummerowia*, *Leucaena*, *Sesbania* and *Medicago* (van Berkum and Eardly, 1998; Sawada et al., 2003).

The species *Sinorhizobium xinjiangense* also clusters fast-growing bacteria that form nitrogen-fixing nodules with soybeans and other legumes, such as *Vigna unguiculata* and *Cajanus cajan* (Chen et al., 1988). They were isolated from soil and soybean nodules collected in Xinjiang province. *S. xinjiangense* strains can be differentiated from those belonging to *S. fredii* by a number of physiological characteristics, such as carbon and nitrogen utilization, intrinsic antibiotic resistance patterns, and ability to grow at acidic or alkaline pH.

Beneficial soil bacteria that by a wide range of mechanisms enhance plant growth are collectively called Plant Growth-Promoting Rhizobacteria (PGPR). The mechanisms by which PGPR promote plant growth are not fully understood, but include among others: (i) the production or change of phytohormone concentrations or balance, such as indolacetic acid, gibberellic acid, cytokinins and ethylene; (ii) asymbiotic N_2 fixation; (iii) antagonism against plant pathogenic microorganisms, such as *Fusarium* spp., by the production of siderophores, beta-1,3 glucanase and chitinases, antibiotics, and cyanide; and (iv) solubilization of mineral phosphates and other nutrients (Cattelan et al., 1999). Frequently, more than one of these plant growth-promoting traits can be exhibited by the same rhizobacterium. In addition to these traits, some rhizobacteria can also promote plant growth indirectly by affecting symbiotic N_2 fixation, nodulation or nodule occupancy. Table IV summarizes different microorganisms and their described effects on soybean growth.

2.1. Mycorrhizal fungi

The roots of almost all higher plants are known to form mutualistic symbioses with fungi. These associations are termed mycorrhizas (fungus roots, from the Greek: mykes = mushroom or fungus and rhiza = root). In 1991 mycorrhiza was defined as "a mutualistic symbiosis between plant and fungus localized in a root or root-like structure in which energy derived from photosynthesis moves primarily from plant to fungus and inorganic resources move from fungus to plant". Mycorrhizas are highly evolved associations between soil fungi and plant roots and they are present in most soils. The partners

Table IV. Diverse soil bacteria and the mechanisms by which soybean growth is enhanced.

Microorganism	Main plant growth- promoting mechanism	Reference
Bradyrhizobium japonicum	N_2 fixation (BNF)	Jordan (1982)
Sinorhizobium fredii	N_2 fixation (BNF)	Albareda et al. (2009b)
Azospirillum	IAA production	Okón and Labandera-Gonzalez (1994)
Pseudomonas sp.	Siderophore production	Fuhrmann and Wollum (1989)
Pseudomonas, *Ralstonia*, *Enterobacter*, *Pantoea, Acinetobacter*	IAA production, solubilization of phosphate	Kuklinsky-Sobral et al. (2004)
Bacillus sp.	Antibiotic production	Li and Alexander (1988)
Streptomyces sp.	Antibiotic production	Li and Alexander (1988)
Serratia sp.	Production of plant growth-regulating compounds	Dashti et al. (1998)

BNF, Biological Nitrogen Fixation; IAA, Indole-3-Acetic Acid.

in this association are members of the fungus kingdom (Basidiomycetes, Ascomycetes and Zygomycetes) and most vascular plants.

In general, the beneficial effects derived from mycorrhizal fungi applications are due to one, or a combination of, the following biological activities: (1) enhancement of the root absorption capacity, mainly due to an increment in the absorption area through the growth of mycorrhizal hyphae, which increases the mobilization and transfer of nutrients (P, N, S, Cu, Zn) from soil to plant; (2) mycorrhizas promote the development of P-solubilizing bacteria around the myco-rhizosphere and enhance the *Rhizobium*-legume symbiosis; (3) antibiotics secreted by mycorrhizae affect root colonization by other microorganisms and decrease plant susceptibility to soil-borne pathogens; (4) the presence of mycorrhizal fungi increases the synthesis of phytohormones, and (5) these may alter soil/plant water relations, which could enhance plant adaptation to extreme situations, such as drought and heavy metal contamination.

Ectomycorrhizal inocula are easy to obtain for nursery applications. At present, however, endomycorrhizal multiplication can only be carried out in the presence of the host plant, which is a constraint factor for increasing the use of endomycorrhizal inocula in agricultural practices. In spite of these limitations, some commercial products have already been sold in the USA. Their most important applications are for: (a) inoculating different substrates (including potting media) and soils that have been fumigated to reduce or eliminate pathogens, like in horticulture crops; (b) reintroduction of plants into degraded places such as mining areas, with extreme pH, metal toxicity, and low organic matter content and fertility, and (c) reintroduction of plants into arid or semi-arid zones (Estaún et al., 2003; Bago et al., 2006; Soriano et al., 2006). Early mycorrhization can contribute not only to plant setting and development but may also increase plant tolerance to biotic and abiotic stress conditions. This inoculation practice has also been applied for micro-propagated vegetal material.

2.2. Azospirillum

Azospirillum brasilense and *A. lipoferum* are being intensively studied because they have been found to enter into a symbiotic relationship with the roots of a wide diversity of tropical grasses and grain crops. Azospirilla are soil-borne microaerophilic α- Proteobacteria able to fix nitrogen. In the past three decades it has become clear that the positive effects of *Azospirillum* on plant growth are not mainly due to biological nitrogen fixation but to the bacterial capacity to produce phytohormones. These bacterial substances, which induce morphological and physiological changes in the inoculated plant root, would lead to an enhancement of water and mineral uptake. This assumption is supported by the observation that the effects of inoculation are highest in fields moderately fertilized with N, P and K, indicating that inoculation with *Azospirillum* does not replace the use of N fertilizers but rather improves their utilization, leading to the same crop productivity at lower levels of fertilization. Hence, *Azospirillum* is a model bacterium for the study of soil microorganisms showing beneficial effects for plant growth in a PGPR manner. Table V summarizes some of the beneficial effects reported for *Azospirillum brasilense* inoculants under greenhouse and/or field conditions.

2.3. Other bacteria showing plant growth-promoting activity

Endophytic bacteria isolated from surface-sterilized soybean root nodules, mostly *Bacillus* sp., were found to increase soybean weight when plants were co-inoculated with *B. japonicum* under nitrogen-free conditions (Bai et al., 2002). Other soybean-associated bacteria (endophytic and epiphytic) showing characteristics related to plant growth promotion have been identified as belonging to the genera *Pseudomonas*, *Ralstonia*, *Enterobacter*, *Pantoea* and *Acinetobacter* (Kuklinsky-Sobral et al., 2004).

These authors reported that the population size of cultivable endophytic bacteria depended on the soybean growth phase, in the order: senescent > flowering > vegetative; and also that the population size and the taxonomic diversity of cultivable epiphytic and endophytic bacteria decreased in agreement with soybean tissues, in the order: roots > stems > leaves.

3. RHIZOBIAL STRAIN SELECTION

Rhizobial strains selected for the production of inoculants must be able to form highly effective nodules with the host

Table V. Beneficial effects derived from inoculation with *Azospirillum brasilense*.

Effects on root hairs and on root architecture	Increasing the number, length and density of mature root hairs
	Shortening of the time of root hair appearance
	Shortening of the distance between the root apex and the region at which root hairs start to elongate
	Shortening of the root elongation zone
	Expansion of the root diameter
	Promotion of nodulation and nitrogen fixation by rhizobial strains
	Enlargement of root cortical cells
	Increase in the number and length of lateral roots
	Elongation of roots by lowering the level of ethylene
Effects on root function	Increase in the total root respiration rates
	Increase in phosphate uptake by roots
	Increase in the capacity of root exudates to induce rhizobial *nod* genes
	Increase in phytohormone content of root tissues
	Increase in the uptake of mineral N, P, Fe
	Enhancement of water uptake capacity and drought tolerance
	Increase in tolerance to salt stress
Effects on plant growth	Increase in leaf area
	Delay of leaf senescence
	Increase in total mineral and carbohydrate content
	Increase in vegetative growth and seed yield

Bean, corn, maize, millet, rice, sorghum, soybean, sugar beet, tomato and wheat are some of the plants in which one or more of the above-mentioned effects have been described.
This table has been constructed from the review published by Dobbelaere et al. (2003).

plant for which they are recommended, and under a wide range of crop conditions.

In addition, if the soil where the crop will be established contains rhizobia strains able to form nodules with the legume crop, the inoculant strain must be competitive with indigenous rhizobia for nodule formation (Date, 2000).

The selection programs of rhizobial strains have several phases:

(1) *Acquisition of nitrogen-fixing bacteria*. They can be obtained from: (i) nodules of field-grown plants, or plants inoculated with soil samples for which there is an interest in obtaining the isolates; (ii) collections of other researchers.
(2) *Purification and authentication of bacterial isolates*. Authentication can be tested by inoculation of the plant concerned with the isolate, followed by re-isolation from a nodule. Nodule isolates should be tested for purity and the ability to nodulate. Isolates showing promising symbiotic capacities in this preliminary screening are stored for further analyses (Lupyawi et al., 2000).
(3) *Evaluation of the symbiotic properties*. Nodulation and effectiveness in N_2 fixation of the isolates is carried out under greenhouse and/or controlled environmental conditions. For these secondary screenings, legume seedlings are aseptically sown in sterilized assemblies, such as test tubes or Leonard jars. Growing seedlings are inoculated and plant responses to inoculation are compared with those of uninoculated controls. In these experiments, a non-inoculated and fertilized control should also be included to show that the legume has the potential to grow well with adequate amounts of N and that growth is not limited by factors other than N (Thompson, 1980; Date, 2000).
(4) *Field trials*. Rhizobial strains showing superior N_2 fixation capacity are then tested under field conditions similar to those where inoculants are intended to be used or applied. Finally, validation of strain performance under a wide spectrum of environmental conditions is also required (O'Hara et al., 2002).

Table VI shows an example of soybean responses to inoculation in soils of SW Spain (Albareda et al., 2009a). Seed yields and seed N content of soybean inoculated with different *S. fredii* strains, such as HH29 or SMH12, were similar to those obtained with the reference strain *Bradyrhizobium japonicum* USDA110 or with uninoculated N-fertilized plots. Thus, *S. fredii* strains HH29, WH1, WH8, WW4 and SMH12 appear to be promising rhizobial strains to be used as soybean inoculants.

Diverse soil microorganisms have also been selected and commercially used as inoculants due to other beneficial effects on plant growth development. These bacterial strains have not been selected by their nitrogen fixation capacity but because they produce phytohormones, enhance mineral uptake and/or act as biocontrol agents of plant pathogens (Bashan, 1998).

Other important characteristic to take into account during the process of strain selection for rhizobia and other beneficial microorganisms is the capacity to exert the beneficial effect under a wide range of field conditions. These bacterial strains should be able to grow in industrial culture media, rising high cell densities, and to survive throughout the process of manufacturing and storage of the inoculants. Persistence in the soil in the absence of the host plant, strain genetic stability, compatibility with agrochemicals and survival under a wide range of soil physical or chemical constraints would be other

Table VI. Nodulation, seed yield and seed nitrogen content of soybean *Glycine max* cv. Osumi inoculated with *Sinorhizobium fredii* strains under field conditions.

Treatment	Nodulation		Yield		
	Number/ plant	Dry weight (mg/plant)	Seed yield (kg/ha)	Seed N (kg/ha)	Harvest Index (%)
WS17	142 ab	653 ab	3245 c	159 c	44 a
S30	121 ab	538 bcd	3319 bc	166 c	42 ab
HH29	163 a	780 a	4338 a	230 ab	43 a
WH1	72 c	524 bcd	4087 ab	226 ab	44 a
WH8	111 bc	527 bcd	4079 ab	229 ab	43 ab
WW4	114 bc	485 cd	4337 a	232 ab	42 ab
SMH12	110 bc	625 abc	4114 a	240 ab	44 a
USDA110	106 bc	441 d	4041 ab	252 a	37 c
T	0.0	0.0	2226 d	91 d	32 d
TN	0.0	0.0	3732 abc	197 bc	40 bc
CV (%)	33.4	24.7	14.4	16.4	5.4

Data are mean values of four replicates. Values followed by the same letter, within each column, are not significantly different at $P < 0.05$. CV: coefficient of variation. T: uninoculated seeds and non-fertilized treatment. TN: uninoculated seeds and N-fertilized treatment. Source: Albareda et al. (2009a). The origin and symbiotic characteristics of the *S. fredii* strains used in this experiment are also described in Rodriguez-Navarro et al. (2003) and Thomas-Oates et al. (2003).

desirable characteristics (Herridge et al., 2002; Obaton et al., 2002; O'Hara et al., 2002).

Obaton et al. (2002) introduced two strains of *Bradyrhizobium japonicum*, recognizable by their intrinsic resistance to high levels of antibiotics and their serological features, into three French calcareous soils under field conditions. These strains were re-isolated 16 or 20 years later and compared with the parental strains kept lyophilized. In the Dijon location, bacterial survival was high although soybean was never grown in the field. However, the introduced *B. japonicum* strains completely disappeared in the Montpellier field after 10 years under vineyard. In the Toulouse field, after the initial soil-introduction of the same two strains as in Montpellier and Toulouse, inoculation of soybean crops (seven years after the soil inoculation) with a new strain enabled this inoculant to occupy 70–80% of the nodules. These results suggest that under some conditions the problem of competition can be solved by repeated inoculation. With regards to agronomic characteristics, there were no important changes in the competitiveness of the strains. Among the eight field isolates tested in a greenhouse for efficiency in comparison with eight lyophilized isolates, seven showed no significant difference for the total weight of soybean or seed yield with the lyophilized isolates. The conclusion of this long-term experiment is that the *B. japonicum* strains used were stable for many characters, but variations in efficiency may occur.

4. TYPES OF INOCULANTS AND QUALITY CONTROL

When a microorganism has been selected as a biofertilizer for agricultural purposes, it is necessary to develop an adequate inoculant formulation to be used under field conditions as a way to obtain equivalent results to those previously obtained under greenhouse conditions (Bashan, 1998). Development of better formulations to ensure survival and activity in the field and compatibility with chemical and biological seed treatments is an area of great interest in the microbial inoculant industry; some approaches include optimization of growth conditions prior to the formulation and development of improved carriers, and the application of this technology in real agronomic practices (Videira et al., 2002).

Nowadays, most solid inoculants are elaborated by mixing bacterial cultures ($>10^9$ viable cells/ml) with powdered or granular carriers, peat being the most commonly used substrate in commercial inoculants (Thompson, 1980).

In general, peat carriers support satisfactory growth and survival of microorganisms during their storage due to their chemical and physical characteristics. However, suitable peat bogs are not available in many countries and peat mining is restricted due to nature conservation politics. Thus, other materials, which are easily available, have also been investigated. Mineral soils (Chao and Alexander, 1984), plant residues (Ferreira and Castro, 2005), clays (Graham-Weiss et al., 1987), perlite (Daza et al., 2000) and wastewater sludge (Rebah et al., 2002) are examples of alternative inoculant carriers. In these carriers bacteria are adsorbed on their surface, but in other formulations, based on polymers, microorganisms are encapsulated and entrapped inside the polymer matrix and the bacteria-carrier mixture is solidified. Several polymers have been investigated and include alginate (Bashan et al., 2002), xanthan gum (Denardin and Freire, 2000) and polyacrylamide gels (Dommergues et al., 1979). These inoculants can be used in powder, granular or bead forms with different moisture content.

Materials used as inoculant carriers must offer some characteristics, such as a high water-holding capacity, chemical and physical uniformity, and pH near neutral or easily adjustable. They should also be non-toxic for the inoculant strains and environmentally safe, and be locally abundant and at a reasonable cost (Stephens and Rask, 2000). However, these properties only indicate the potential for a good carrier because the final selection must be based on biological assays, such as

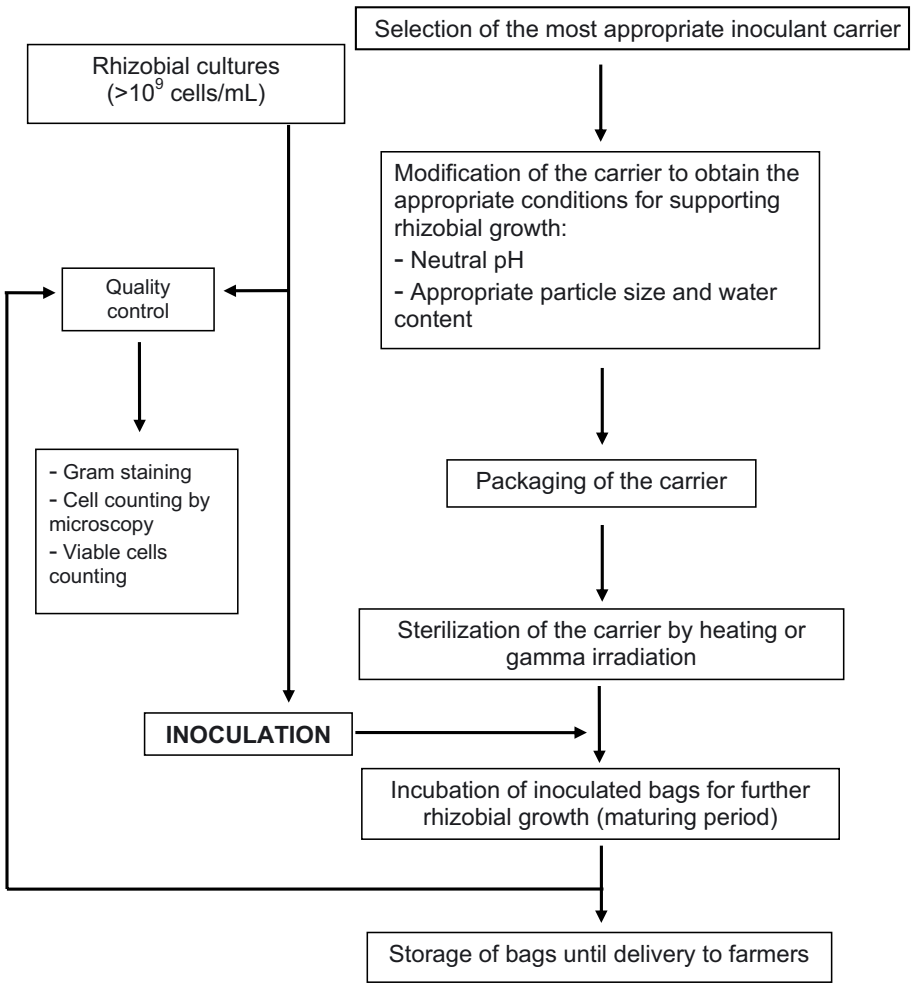


Figure 1. Scheme of solid inoculant production.

rhizobia growth and survival during storage (Albareda et al., 2009b).

Various types of containers were used to package the inoculant in the early days of the inoculant industry. They included glass bottles, metal cans and rigid plastic containers.

Flexible low-density polyethylene film (0.038–0.076 mm) is used almost everywhere nowadays. This material provides high moisture retention, sufficient gas exchange and can be heat-sealed.

Polyethylene bags that can be autoclaved are commercially available. Thus, packaged peat units can be autoclaved for 50 min at 121 °C. Afterwards, the inoculation of individual pre-sterilized bags can be accomplished by injection or by using an automatic dispenser apparatus. Inoculated bags are incubated at the appropriate temperature to allow further growth of rhizobia. Figure 1 shows a simplified scheme of the procedure most frequently used in the production of solid-based inoculants.

Successful production of rhizobial inoculants needs to be associated with an effective, regulatory Quality Control program (Thompson, 1980; Herridge et al., 2002). The whole question of inoculant production and use starts with quality; if the quality is poor the rest is irrelevant. Several inoculant quality surveys found that 50–90% of the inoculants had less than 10^8 viable rhizobia per gram of carrier. The numbers of rhizobia in the inoculants were inversely related to the numbers of microbial contaminants, although most of the inoculants sampled were produced with sterile carriers, indicating problems with the production and factory-level quality control. All these data reinforced the need for regulation and enforcement of inoculant quality standards. Regardless of whether public or private sectors produce the inoculants, it is highly advisable that quality standards are controlled by a Central Agency such as those operating in Australia, France and Brazil.

Product quality involves prior checking of strain efficacy for symbiotic performance before inoculant production starts on a commercial scale. Purity of rhizobial culture should be ensured before its addition to the carrier. This can be done by microscopy observation and Gram-staining. Determination of the culture pH may also help to reveal the presence of contaminants. Sterility of the carrier before mixing with the rhizobial culture can be checked by plating carrier dilutions

on appropriate media and checking for the presence of microbial contaminants. Moisture content of the final product and rhizobial density have to be determined. Packaging controls, such as verification of proper bag sealing, lot number, expiration date and weight of the filled bags are also standards of the quality control checks.

The regulatory authorities of some countries have set minimum standards for rhizobial numbers. Inoculant manufacturers may apply these standards voluntarily. The quality of inoculants is as dependent on factors and conditions that occur after packaging as it is on those that occur during manufacturing, transportation, and in the distribution network. All of these factors together account for the shelf life of the product. Extended maturing periods and low storage temperature are considered relevant factors in providing the maximum population and in reducing rhizobial population decline on seeds. Inoculants are generally manufactured just before the planting season. The survival of rhizobia on the seed surface is lower than on solid carriers due to the lack of protection against desiccation, high temperature, and/or toxic compounds on the seed coat. This is an important aspect since the viable number of rhizobia applied to the seed is of crucial importance for successful root colonization and nodulation (Hume and Blair, 1992).

Liquid inoculants are industrial formulations that simplify the production process. As there is no need to prepare and amend a carrier, the application to seeds or fields is easier. In addition, these formulations are usually composed of low-cost materials and offer an attractive option to the unavailability of other possible carriers. However, bacterial survival in this type of inoculant and on inoculated seed is usually not as good as in carrier-based inoculants because there is not a carrier that protects the cells (Singleton et al., 2002; Tittabutr et al., 2007). To overcome these problems, liquid formulations consist of broth cultures amended with agents that enhance cell survival during the storage. They also protect cells after the inoculant application on seeds or soil, where stress conditions such as high temperature or desiccation can occur. These additives can include wetting and dispersal agents, nutrients, compounds that bind, and inactivate soluble toxic seed-coat compounds or adhesives (Singleton et al., 2002).

Other liquid formulations include oil-based products, rhizobia concentrates or freeze-dried cultures that are diluted in water or liquid suspension before use (Thompson, 1980). Inoculants can be applied after adding the product (additive) onto pre-inoculated seeds, which in this form can be stored before sale. More common is the application of inoculants to seeds at sowing or to the furrow. When inoculants are placed directly into the furrow, liquid or granular inoculants are preferentially used.

5. THE COMPETITION PROBLEM

An important factor that can limit nodule occupancy by symbiotically-superior inoculant strains is the presence of specific rhizobial populations in the soil that, being well adapted to soil conditions, are poorly effective in nitrogen fixation. Thus, indigenous rhizobia in the soil impose a competition barrier to the establishment of inoculant strains, possibly leading to inoculation failure.

In these circumstances, predicting nodule occupancy by rhizobia introduced into different environments becomes a key factor for a more judicious use of rhizobial inoculants. Thies et al. (1991a, b) developed mathematical models which could be used to predict the success of rhizobial inoculation in diverse environments. These models are based on indices of the size of indigenous rhizobial populations and on the availability of mineral N. The fact that 59% of the observed variation in inoculation responses could be due to plant responses to indigenous rhizobia illustrates the profound influence of the size of soil rhizobial populations on the successful use of rhizobial inoculants. The responses to inoculation and the competitive success of inoculant rhizobia were inversely related to numbers of indigenous rhizobia.

Nodulation could also be limited in soils devoid of homologous rhizobia of the legume crop that will be sown, even when using elite inoculant strains. These constraints to the successful nodulation of inoculants illustrate the difficulties of the newly introduced inoculant in overcoming detrimental interactions with environmental factors, such as high nitrate content of the soil, inadequate temperature (Zhang et al., 1997), drought conditions and calcareous soils (Hungria and Vargas, 2000; Zahran, 1999). In addition, variations in nodulation and N_2-fixation efficiency frequently occur in a bacteria strain-/legume cultivar-specific manner (Sanginga et al., 2000).

Table VII shows an example of the soybean *cultivar effect* on the indigenous soybean rhizobia populations and on the soybean nodule occupancy by fast- and slow-growing rhizobia in two Chinese provinces. The estimated levels of the total indigenous soybean rhizobia population of Xinjiang soil were clearly higher when soybean cultivar Jing Dou 19 was used than those estimated with cultivar Heinong 33. The percentage of fast-growing rhizobia recovered from soybean nodules was high in both soil samples. Interestingly, this prevalence of fast-growing soybean rhizobia strains on nodules of soybean plants inoculated with Xinjiang soil was only observed for the Asiatic soybean cultivars.

6. MOLECULAR ASPECTS OF THE RHIZOBIA-SOYBEAN SYMBIOSIS

6.1. Nodulation genes and Nod factors

Nodulation genes are defined as those rhizobial genes which play a role in nodulation or which are co-ordinately regulated with such genes. In chronological order of discovery they were designated as *nod*, *nol* and *noe*. The identification of nodulation genes was advanced enormously by the discovery that, in *Sinorhizobium meliloti* and *Rhizobium leguminosarum*, many of the nodulation genes are plasmid-borne. These plasmids, carrying nodulation and nitrogen-fixation genes, are called symbiotic plasmids or pSym. Rhizobial strains cured of their pSym are unable to nodulate unless a homologous pSym plasmid is reintroduced by conjugation. Also, in certain cases,

Table VII. Effect of the soybean cultivar on the isolation of soybean microsymbionts (fast-growing soybean rhizobia and slow-growing soybean rhizobia) from Chinese soil samples.

Province of soil sample origin	Soybean cultivar	MPN ($\times 10^4$)[a]	Ratio FSR/SSR (%)[b]
Henan	Heinong 33	4–50	73/27[a]
	Linzhen		71/29[a]
	Williams	2–30	65/35[a]
	Bragg		68/32[a]
Xinjiang	Jing Dou 19	4–51	100/0
	Heinong 33	0.06–0.8	99/1
	Williams		8/92
	Kobe		7/93

[a] The number of soybean rhizobia in each soil sample was estimated by the Most Probable Number technique.

[b] Statistical analyses were performed with cultivars inoculated with a Henan soil sample. Numbers in the same column followed by the same letter are not significantly different ($P < 0.05$) using the Fisher test for comparing proportions. For the Xinjiang sample statistical analysis was not carried out since the numbers are very different.

Source: Yang et al. (2001).
FSR, Fast soybean rhizobia. SSR, Slow soybean rhizobia. MPN, Most Probable Number.
Heinong 33, Linzhen and Jing Dou 19 are Asiatic soybean cultivars.
Williams, Bragg and Kobe are American soybean cultivars.

Table VIII. Most relevant *Bradyrhizobium japonicum* and *Sinorhizobium fredii* nodulation genes (Downie, 1998).

Nodulation genes	*B. japonicum*	*S. fredii*	Function of gene product
nodA, nodB, nodC	+	+	Biosynthesis of the Nod factor backbone
nodD1, nodD2	+	+	Regulator
nodI nodJ	+	+	Nod factor secretion
nodS, nodU	+	Present but non-functional	Modification of Nod factors
nodV, nodW, nolA	+	-	Regulator
nodZ, nolK, noeL	+	+	Modification of Nod factors
nolM, nolN, nolY, noeD	+	-	Undefined

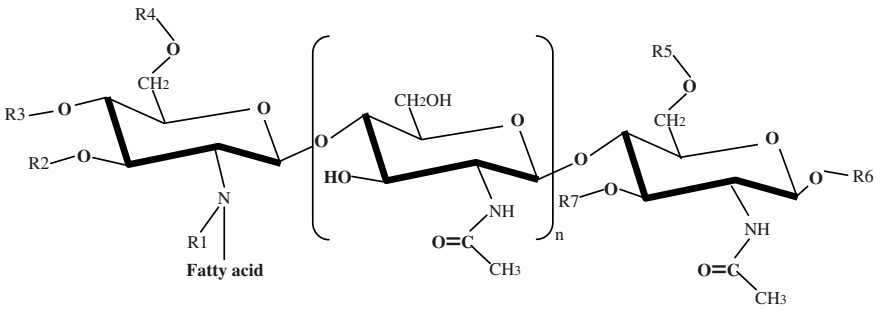
intraspecific transfer of symbiotic plasmids can originate rhizobial transconjugants that have gained the capacity to nodulate new legume hosts.

To form nitrogen-fixing nodules, rhizobia have to multiply in the rhizosphere, attach to legume root hairs, penetrate into the root through infection threads, infect the cytoplasm of specialized root-nodule cells, and change into the bacteroid forms. Bacteroids are the rhizobial symbiotic form able to fix nitrogen. Like in organs being formed by cells of any particular organism, leguminous plant cells and rhizobia must establish a bidirectional communication in order to form a highly structured and functional root-nodule organ. This communication, which is carried out by chemical signals, starts from the very beginning of the microbe-plant interaction, when rhizobia are outside the root, and has to continue during the whole process of nodule morphogenesis.

Rhizobia produce lipo-chitin oligosaccharide signal molecules, called LCOs or Nod factors, in response to plant-produced flavonoid compounds (Fisher and Long, 1992). Flavonoids that induce the transcription of rhizobial *nod* genes include chalcones, flavanones, flavones, flavonols, isoflavonoids, coumestans and anthocyanidin. All these flavonoids are derived from the phenylpropanoid molecules that enter the flavonoid pathway through chalcone synthase (Schlaman et al., 1998). The most abundant flavonoids produced by soybean roots are the isoflavones daidzein and genistein, and coumestrol (Kosslak et al., 1987; D'Arcy-Lameta, 1986). The molecular dialogue between the two symbionts, the legume and *Rhizobium*, starts when flavonoids exuded by the legume root interact with the bacterial protein NodD. This flavonoid-NodD interaction provokes the transcriptional activation of rhizobial nodulation genes. Expression of nodulation genes ultimately results in the production and secretion of Nod factors which in turn induce hair curling and nodule meristem initiation in the plant root (Gage, 2004; Jones et al., 2007).

Nodulation genes (*nod*, *nol* and *noe*) can be subdivided into a number of different categories: these include those that are involved in: (i) regulation of other nodulation genes; (ii) biosynthesis and modification of Nod factors; (iii) nod factor secretion; (iv) protein secretion; and (v) genes of undefined function. Table VIII lists different nodulation genes described in the soybean symbionts *Bradyrhizobium japonicum* and *Sinorhizobium fredii*.

The chemical structure of rhizobial LCOs consists of a backbone of two to six β-(1→4)-linked *N*-acetyl-glucosamine (GlcNAc) residues bearing an amide-bound fatty acyl residue, saturated or unsaturated, on the non-reducing terminal GlcNAc residue. This basic structure shows variations among the different rhizobial species and contributes to determining bacterial host specificity. For instance, the C-6 of the reducing GlcNAc residue may either be unsubstituted, sulfated,



Rhizobia	Fatty acid	R1	R2	R3	R4	R5	R6	R7	*n*
S. fredii	$C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$ $C_{20:0}$, $C_{20:1}$ $C_{20:3}$	H	H	H	H	MeFuc Fuc	H	H	1,2,3
B. japonicum	$C_{16:0}$, $C_{16:1}$, $C_{18:1}$	H	H	H	H Ac	MeFuc	H	H	3
B. elkanii	$C_{16:0}$, $C_{18:1}$	H Me	Ac Cb	H Ac Cb	H Ac Cb	MeFuc Fuc	H Gro	H	2,3

Ac: *O*-acetyl; Cb: *O*-carbamoyl; Fuc: fucosyl; MeFuc: 2-*O*-methylfucosyl; Gro: glyceryl; n: number of internal GlcNac residues. This table was constructed from D'Haeze and Holsters (2002) and Thomas-Oates et al. (2003).

Figure 2. Nodulation factors produced by *Sinorhizobium fredii*, *Bradyrhizobium japonicum* and *B. elkanii*.

acetylated, fucosylated, acetylfucosylated, sulfofucosylated or 2-*O*-methylfucosylated. A summary of the LCOs produced by *B. japonicum, B. elkani* and *S. fredii* is shown in Figure 2. The diversity of LCOs produced by *S. fredii* strains (Bec-Ferté et al., 1994; Gil-Serrano et al., 1997; Thomas-Oates et al., 2003) appears to be wider than that produced by *B. japonicum* (Carlson et al., 1993; Sanjuan et al., 1992) or *B. elkanii* (Carlson et al., 1993; Stokkermans et al., 1996). This difference, however, might simply reflect the improvements of mass spectrometry technology achieved in the last two decades, so that the structure of *S. fredii* LCOs was determined by using more sensitive equipment.

Rhizobial LCOs induce many early nodulation events that take place at the epidermal surface, cortex and pericycle of legume roots. Some of these root responses to the presence of LCOs are: curling, deformation, branching and swelling of root hairs, depolarization of the membrane potential of plant cells, formation of preinfection threads, formation of nodule primordia in the inner or outer cortex, and induction of cell cycle genes (Masson-Boivin, 2009).

In addition to Nod factors, different rhizobial surface polysaccharides are required for successful nodulation, during which they might act as signal molecules or could prevent plant defence reactions (Becker and Pühler, 1998; Breedveld and Miller, 1998; Fraysse et al., 2003; Mathis et al., 2005). Exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS or K-antigens) and cyclic β-glucans (CGs) are the main rhizobial polysaccharides investigated for their roles in nodulation (Fraysse et al., 2003; Parada et al., 2006). The importance of each particular polysaccharide in the nodulation process varies according to the type of nodule (determinate or indeterminate) that each particular legume is able to develop. For instance, EPS is required for the formation of nitrogen-fixing nodules on plants forming indeterminate nodules, such as *Medicago sativa* (alfalfa), but apparently it is dispensable in plants that form determinate nodules, such as *Glycine max* (soybean). In contrast, the symbiotic impairment of rhizobial mutants affected in LPS production appears to be severer in determinate nodule-forming legumes than in those forming indeterminate nodules (Fraysse et al., 2003; Gage, 2004). Here, we will give a brief description of the structures of these four surface polysaccharides of the soybean symbionts *S. fredii* and *B. japonicum,* as well as their implication in the nodulation process.

6.2. Cyclic glucans (CG)

Cyclic glucans produced by *Agrobacterium tumefaciens*, all biovars of *Rhizobium leguminosarum*, *S. meliloti* and *S. fredii* HH103 only contain glucosyl residues that are solely linked by β-(1, 2) glycosidic bonds. Cyclic glucans produced by *S. fredii* HH103 are composed of 18–24 glucosyl residues without or with 1-phosphoglycerol as the only substituent

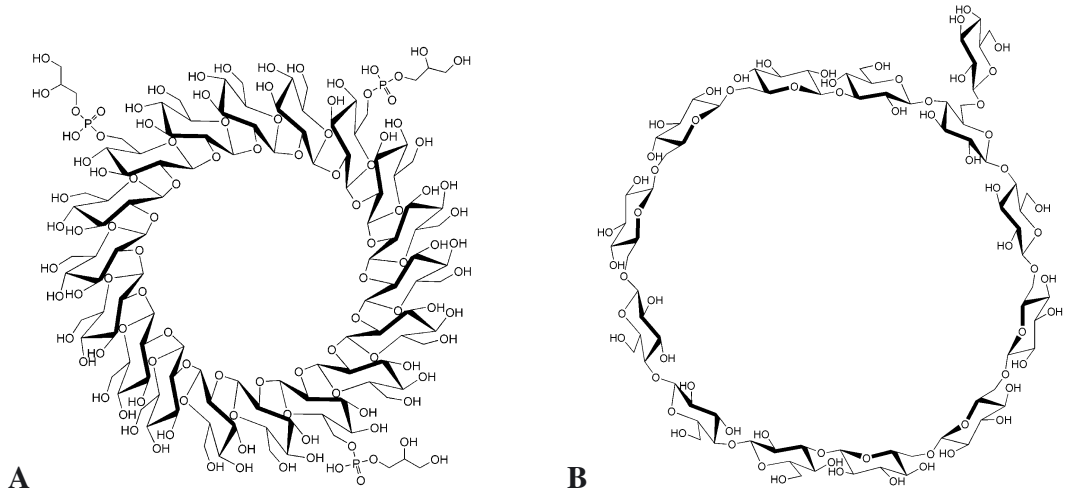


Figure 3. Structures of the *S. fredii* HH103 (A) and *B. japonicum* USDA110 (B) cyclic glucans.

(Fig. 3A). Cyclic glucans produced by species of *Bradyrhizobium japonicum* contain both β-(1, 3) and β-(1, 6) glycosidic linkages (Breedveld and Miller, 1998). These cyclic glucans contain 10 to 13 glucosyl residues and appear to be branched in structure. The structure of the bradyrhizobial β-(1,3)-β-(1, 6) cyclic glucan, which is composed of 13 glycosil residues, has been proposed to consist of a backbone of 12 glucose residues containing triplets of β-(1, 3)-linked residues separated by triplets of β-(1, 6)-linked glucose residues. The remaining glucose residue would be present as a branch on the C-6 of a β-(1, 3)-linked glucosyl residue (Fig. 3B).

In *S. meliloti*, two genes, *ndvA* and *ndvB*, are necessary for cyclic glucan production (Breedveld and Miller, 1998). The *ndvA* gene is involved in cyclic glucan transport, while *ndvB* [renamed *cgs* (cyclic glucan synthase)] is responsible for the biosynthesis of cyclic glucans. *Medicago sativa* plants inoculated with *S. meliloti ndvB,* mutants unable to produce cyclic glucans only form ineffective pseudonodules, which contain a small number of infection threads that abort at early stages (Breedveld and Miller, 1998).

Similarly, a *S. fredii* HH103 *cgs* mutant is unable to produced cyclic glucans and failed to induce the formation of nitrogen-fixing nodules in any of the legumes tested, regardless of whether it formed determinate (*Glycine max* and *V. unguiculata*) or indeterminate (*Glycyrrhiza uralensis*) nodules. This *S. fredii* HH103 *cgs* mutant also shows alterations of other bacterial traits (Tab. IX). Exopolysaccharides (EPS) produced by the *cgs* mutant are clearly altered. The motility and speed of growth in the hypoosmotic GYM medium is also negatively affected by the *cgs* mutation (Crespo-Rivas et al., 2009).

Bradyrhizobium japonicum mutants unable to produce cyclic glucans also form ineffective nodules, although in this case soybean nodules contain bacteroids (Bhagwat and Keister, 1995). In summary, mutations that abolish cyclic glucan production are extremely harmful from a symbiotic point of view since rhizobial nodulation is severely impaired in determinate and indeterminate nodule-forming legumes. The symbiotic impairment observed in rhizobial *cgs* mutants might be due to a dramatic alteration in the bacterial surface properties. However, studies using *S. meliloti* pseudorevertants indicate that cyclic glucans, by themselves, might not be strictly required for the successful interaction of symbiotic bacteria with their eukaryotic host. Neither *S. meliloti* pseudorevertants selected for restoration of motility, nor those selected for nodulation capacity, regained the capacity to produce cyclic glucans, which indicates that this polymer is not strictly required for nodule development, even though *S. meliloti cgs* mutants only form pseudonodules that do not fix nitrogen (Dylan et al., 1990).

The fact that *S. fredii* HH103 *cgs* mutants show a pleiotropic phenotype makes it difficult to determine the relative symbiotic importance of each particular phenotypic change observed. Nevertheless, the recent finding that the application of purified cyclic glucans from the phytopathogen *Xanthomonas. campestris* to *Nicotiana benthamiana* leaves provokes the suppression of different plant defence mechanisms clearly indicates that cyclic glucans, by themselves, play an important role in plant-microbe interactions (Rigano et al., 2007).

6.3. K-antigen polysaccharides (KPS)

The capsular polysaccharide (KPS) surrounds the bacterium and forms a hydrated matrix which confers resistance to bacteriophages and to the dry conditions often present in the rhizosphere environment. The structures of KPS produced by different *S. meliloti* and *S. fredii* strains have been determined (Reuhs et al., 1993; Reuhs et al., 1998; Gil-Serrano et al., 1999; Rodríguez-Carvajal et al., 2001; Rodríguez-Carvajal et al., 2005). Although the KPS structure is strain-specific, most *Sinorhizobium* K-antigens contain Kdo or related compounds (Kdx), such as derivatives of the 3-deoxy-non-2-ulosonic acid (Kdn). In *S. meliloti* and *S. fredii*, the KPS repeating unit frequently shows a conserved structural motif involving a hexose linked to Kdx. Some *S. fredii* strains,

Table IX. Alterations caused by mutation in the *cgs* gene of *S. fredii* HH103 (Crespo-Rivas et al., 2009).

Bacterial trait	Wild-type *S. fredii* HH103	*S. fredii* HH103 *cgs* mutant
Production of CG	+	–
Estimated generation time in yeast-extract mannitol broth (YMB)	204 min	228 min
Estimated generation time in the hypoosmotic GYM medium	240	324
Mobility in GYM medium	+	–
Amount of EPS produced in GYM	+	+++
Molecular weight of EPS produced in GYM	Most of 50–60 kDa	50–60 kDa and also higher than 2000 kDa
Level of acetate and pyruvate substituents in the EPS produced in GYM	+	+++
Expression of *exoA,* a gene involved in EPS biosynthesis	+	+++
Symbiotic capacity with soybean cultivars McCall, Osumi, Williams and Peking, and *Glycyrrhiza uralensis*	Nitrogen-fixing nodules are formed	Only pseudonodules devoid of bacteria and unable to fix nitrogen are formed.

CG, β-(1, 2) cyclic glucans; EPS, Exopolysaccharides; GYM, a hypoosmotic medium composed of sodium glutamate (1 mM), yeast extract (0.2 g/L), mannitol (1 mM), $CaCl_2$ (0.5 mM), $MgSO_4$ (0.5 mM) and K_2HPO_4 (1 mM); YMB, yeast-extract mannitol broth.

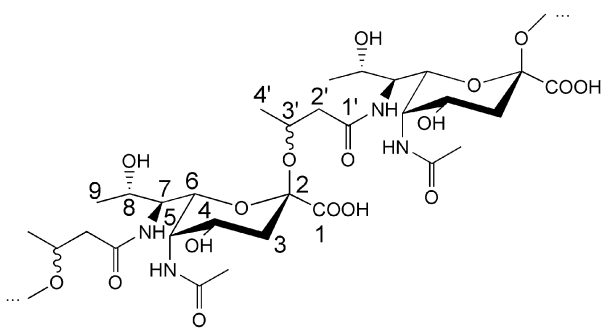


Figure 4. Chemical structure of two repeating subunits of the capsular polysaccharide (KPS) produced by *S. fredii* HH103 (Gil-Serrano et al., 1999).

however, produce a KPS whose structure differs from the hexose-Kdx consensus motif. This is the case of *S. fredii* HH103, which produces a KPS consisting of a homopolymer of a pseudaminic acid derivative (Fig. 4). Curiously, all the *S. fredii* strains so far investigated that produce KPS in which the hexose-Kdx repeating unit is not present are also able to induce the formation of nitrogen-fixing nodules in both American and Asiatic soybean varieties (Reuhs et al., 1998; Rodríguez-Carvajal et al., 2005). In contrast, the *S. fredii* strains whose KPS present the hexose-Kdx consensus motif effectively nodulate Asiatic soybeans but fail to fix nitrogen with American soybean cultivars (Keyser et al., 1982; Reuhs et al., 1998). Thus, KPS could be involved in determining the symbiotic compatibility of the *Sinorhizobium fredii* strains with the different soybean cultivars, a phenomenon called "cultivar-strain specificity".

Rhizobial genes involved in KPS were first described in *S. meliloti* Rm41. In this alfalfa symbiont, three different gene clusters required for K-antigen production have been identified and named *rkp-1*, *rkp-2* and *rkp-3* (Petrovics et al., 1993; Kereszt et al., 1998; Kiss el al., 2001). These three distinct *rkp* regions have also been identified in *S. fredii* HH103, although notable variations have been found. Information about rhizobial genes involved in KPS production is only available for two *S. meliloti* strains (Rm41 and 1021), for *Rhizobium* sp NGR234, and for *S. fredii* HH103.

The *S. meliloti* Rm41 *rkp-1* region contains 10 genes, denominated *rkpA* to *rkpJ* (Fig. 5), whose encoded products may participate in the synthesis, modification and transfer of a putative lipophilic molecule that might act as a specific lipid carrier during KPS biosynthesis and/or as the lipidic anchor to the outer membrane of the bacterium. The *rkpA-rkpJ* genes are flanked by two genes named *rkpU* and *KpsF3*. In *S. meliloti* 1021 and *S. fredii* HH103, the *rkpABCDEF* genes (as defined in *S. meliloti* Rm41) are fused into a single open reading frame, named *rkpA*, which encodes a long polypeptide of 2504 and 2500 residues, respectively (Fig. 5).

The *rkp-2* region of *S. meliloti* strains Rm41 and 1021 contains two genes, *lpsL* and *rkpK*, which code for enzymes involved in the metabolism of nucleotide diphosphosugars. Both genes are required for wild-type lipopolysaccharide (LPS) production. The product of the *rkpK* gene, a UDP-glucose dehydrogenase, is also required for KPS production. These two genes are also present in *S. fredii* HH103, although the isolation of mutants affected in *lpsL* and *rkpK* has not been reported.

The *rkp-3* region of *S. meliloti* strain Rm41 is constituted by eleven genes. They are denominated *rkpL* to *rkpY*. The *rkpR*, *rkpS* and *rkpT* genes (which are involved in KPS export) and the *rkpZ* (a chain-length determinant, formerly known as *lpsZ*) are located in the 3' part of this region. The *S. meliloti* 1021 genome lacks the *rkpLMNOPQ* genes, which are present in the 5' part of the Rm41 *rkp-3* region (for a review, see Becker et al., 2005). These six genes are also present in *S. fredii* HH103 and code for enzymes required for the biosynthesis of pseudoaminic acid, which is present in the repeating units of the KPS of Rm41 and HH103 but not in that of strain 1021.

In *S. meliloti* strains, KPS can replace exopolysaccharides (EPS) for a successful nodulation with *Medicago sativa* (alfalfa). Due to this symbiotic equivalence between EPS and KPS, *S. meliloti* AK631 (an *exoB* mutant derivative of *S. meliloti* Rm41 able to produce KPS but not EPS) is able to form nitrogen-fixing nodules with alfalfa. EPS mutants of *S. meliloti* 1021 are symbiotically impaired because the KPS

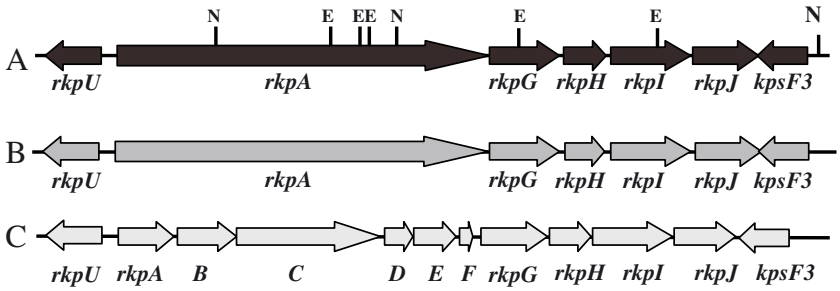


Figure 5. Genetic organization of the *rkp-1* region of (A) *S. fredii* HH103; (B) *S. meliloti* 1021; (C) *S. meliloti* Rm41.

Figure 6. Nodules (white arrows) and pseudonodules induced on roots of the soybean cultivar Williams by a *S. fredii* HH103 *rkpI* mutant.

produced by this strain is symbiotically inefficient. *S. fredii* HH103 mutants affected in *rkp* genes belonging to the *rkp-1* and *rkp-3* regions are severely impaired to nodulate *Glycine max* and *Cajanus cajan* (pigeon pea). Soybean and pigeon pea plants inoculated with these HH103 *rkp* mutants showed reduced nodulation (including the formation of pseudonodules unable to fix nitrogen) and severe symptoms of nitrogen starvation (Fig. 6).

S. fredii HH103 mutants unable to produce EPS (by mutation in the *exoA* gene) do not show any reduction of their symbiotic capacity with soybean. Thus, KPS, but not EPS, is of crucial importance for the symbiotic capacity of *S. fredii* HH103. In contrast to the situation described in *S. meliloti*, EPS and KPS of *S. fredii* HH103 are not symbiotically equivalent, because mutants in *rkp* genes are symbiotically impaired regardless of whether or not EPS is produced.

Although it is not fully understood why KPS is relevant for the *S. fredii* HH103 symbiotic capacity with soybeans or how it can functionally replace EPS in the *S. meliloti*-alfalfa symbiosis, experimental evidence indicates that KPS could contribute in two different ways to the successful establishment of functional nodules: by providing a passive protection barrier against plant substances that could be harmful to the bacteria and by promoting infection thread initiation and development (Pellock et al., 2000; Reuhs et al., 1993).

6.4. Lipopolysaccharides (LPS)

Lipopolysaccharides are located in the outer leaflet of almost all Gram-negative bacteria. These glycoconjugate macromolecules play relevant roles in the *Rhizobium*-legume symbiosis: LPS protect rhizobia from plant-derived antimicrobial compounds, are involved in plant recognition and adhesion to the plant, and appear to promote infection and nodule invasion (Kannenberg et al., 1998). In general, LPS consist of two different moieties: the lipid A, a glycolipid located in the outer membrane, and a saccharide portion that extends towards the outside of the cell. This saccharide part consists of two well-defined parts: an oligosaccharide part, called the *core*, and an O-specific polysaccharide showing antigenic properties.

Only the complete structure of the LPS produced by *Rhizobium etli*, a symbiont of *Phaseolus vulgaris*, is well known (Forsberg and Carlson, 1998; Kannenberg et al., 1998; De Castro et al., 2008). The sugar and lipid composition of the LPS produced by *Bradyrhizobium japonicum* USDA110 has also been reported (Puvanesarajah et al., 1987). *Bradyrhizobium japonicum* USDA110 mutants unable to synthesize the O-antigen region are severely impaired to nodulate soybeans. To our knowledge, information about sugar and lipid composition of the LPS produced by *Sinorhizobium fredii* lipopolysaccharides is not available. LPS produced by *S. fredii* HH103 *lpsB* mutants show alterations in their electrophoretic profile. These mutants are severely impaired to nodulate soybean and other legumes. Thus, the *S. fredii* HH103 LPS appears to be of crucial importance for the bacterial symbiotic capacity.

6.5. Exopolysaccharides (EPS)

The structure of the EPS produced by *Bradyrhizobium japonicum* USDA110 has been determined. This EPS contains glucose, mannose, galactose, galacturonic acid and 4-O-methylglucose (Becker and Pühler, 1998). The chemical structure of the *S. fredii* EPS has not been determined yet. *B. japonicum* and *S. fredii* mutants unable to produce EPS form nitrogen-fixing nodules on soybean (Becker and Pühler, 1998; Parada et al., 2006). Although EPS does not seem to be essential for the formation of nodules on this legume, different reports have shown that early stages of the development of soybean nodules induced by *B. japonicum* EPS mutants are disturbed (Becker and Pühler, 1998).

7. CONCLUSIONS

Soybean cropping relying on biological nitrogen fixation can produce similar or higher yields than those receiving N fertilizers. There exists a wide positive response to the agronomic inoculation practice which goes back one century in countries such as the USA, and is more recent in Brazil, Argentina and Canada. Commercial inoculants of different types are market-available and inoculation of legumes is economically feasible. At present, with the growing interest in optimizing agriculture, to reduce its environmental impact and enhance biodiversity, it should be of great concern that future work on breeding new soybean cultivars takes into account the N_2 fixation potential with selected rhizobia strains, which provides competitive yields and a positive N balance for the soil.

Nodulation factors and bacterial surface polysaccharides can act as signal molecules in the (*Brady/Sino*) *rhizobium*-soybean symbiotic interaction. The chemical structures of the signals produced by *Bradyrhizobium japonicum* strains are not exactly the same as those produced by *Sinorhizobium fredii*. Moreover, *B. japonicum* does not produce K- antigen polysaccharides, while it is relevant for the *S. fredii*-soybean symbiosis. All these facts indicate that although Nodulation factors, cyclic glucans or lipopolysaccharides are required for the formation of nitrogen-fixing nodules on soybean roots, the level of "symbiotic fitness" between both partners might be due to the effects of the particular cocktail of signal molecules produced by each particular soybean rhizobial strain. How these signals are perceived by each particular soybean cultivar will also contribute to the final outcome of the symbiotic interaction.

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