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REVISÃO DE LITERATURA

EXOPOLYSACCHARIDES PRODUCED BY THE SYMBIOTIC NITROGEN-FIXING BACTERIA OF LEGUMINOSAE[1]

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SUMMARY

The process of biological nitrogen fixation (BNF), performed by symbiotic nitrogen fixing bacteria with legume species, commonly known as α and β rhizobia, provides high sustainability for the ecosystems. Its management as a biotechnology is well succeeded for improving crop yields. A remarkable example of this success is the inoculation of Brazilian soybeans with *Bradyrhizobium* strains. Rhizobia produce a wide diversity of chemical structures of exopolysaccharides (EPS). Although the role of EPS is relatively well studied in the process of BNF, their economic and environmental potential is not yet explored. These EPS are mostly species-specific heteropolysaccharides, which can vary according to the composition of sugars, their linkages in a single subunit, the repeating unit size and the degree of polymerization. Studies have showed that the EPS produced by rhizobia play an important role in the invasion process, infection threads formation, bacteroid and nodule development and plant defense response. These EPS also confer protection to these bacteria when exposed to environmental stresses. In general, strains of rhizobia that produce greater amounts of EPS are more tolerant to adverse conditions when compared with strains that produce less. Moreover, it is known that the EPS produced by microorganisms are widely used in various industrial activities. These compounds, also called biopolymers, provide a valid alternative for the commonly used in food industry through the development of products with identical properties or with better rheological characteristics, which can be used for new applications. The microbial EPS are also able to increase the adhesion of soil particles favoring the mechanical stability of aggregates, increasing

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levels of water retention and air flows in this environment. Due to the importance of EPS, in this review we discuss the role of these compounds in the process of BNF, in the adaptation of rhizobia to environmental stresses and in the process of soil aggregation. The possible applications of these biopolymers in industry are also discussed.

Index terms: Exopolysaccharides, nodules, environmental stress, soil aggregation, rhizobia.

RESUMO: *EXOPOLISSACARÍDEOS PRODUZIDOS POR BACTÉRIAS FIXADORAS DE NITROGÊNIO SIMBIÓTICAS DE LEGUMINOSAE*

O processo de fixação biológica de nitrogênio (FBN), realizado por bactérias fixadoras de N_2 simbióticas de leguminosas, comumente denominados α e β rizóbios, proporciona alta sustentabilidade aos ecossistemas. Seu manejo como uma biotecnologia é bem sucedido para aumentar a produtividade das culturas. Um exemplo notável desse sucesso é a inoculação da soja com estirpes de Bradyrhizobium.*Os rizóbios produzem grande diversidade de estruturas químicas dos exopolissacarídeos (EPS). Embora o papel dos EPS seja relativamente bem estudado no processo de FBN, o seu potencial econômico e ambiental ainda não é explorado. Esses EPS são principalmente heteropolissacarídeos espécie-específicos, que podem variar de acordo com a composição dos açúcares, as suas ligações em uma única subunidade, o tamanho da unidade repetitiva e o grau de polimerização. Estudos mostram que os EPS produzidos por essas bactérias exercem importante papel no processo de invasão, formação do cordão de infecção, desenvolvimento do bacteroide e do nódulo e resposta de defesa da planta. Esses EPS também conferem proteção a essas bactérias quando submetidas a diversos estresses ambientais. Em geral, estirpes de rizóbios que produzem maior quantidade de EPS são mais tolerantes às condições adversas, quando comparadas com estirpes que produzem menor quantidade. Além disso, sabe-se que os EPS produzidos por microrganismos são amplamente utilizados em vários segmentos industriais. Esses compostos, também denominados biopolímeros, fornecem uma alternativa válida para a substituição das gomas comumente usadas na indústria de alimentos, por meio do desenvolvimento de produtos com propriedades praticamente idênticas ou com melhores características reológicas, que podem ser usados para novas aplicações. Os EPS microbianos também são capazes de aumentar a adesão de partículas do solo, favorecendo a estabilidade mecânica dos agregados, além de aumentarem os níveis de retenção de água e fluxo de ar nesse ambiente. Diante da importância dos EPS, na presente revisão discute-se o papel desses compostos no processo de fixação biológica de N_2, na adaptação dos rizóbios a estresses ambientais, bem como no processo de agregação do solo. As possíveis aplicações desses biopolímeros na indústria também são discutidas.*

Termos de indexação: exopolissacarídeos, nodulação, estresse ambiental, agregação do solo, rizóbios.

INTRODUCTION

Nitrogen (N) is a constituent of various cellular components, such as amino acids, proteins, enzymes, nucleic acids and chlorophyll. Numerous fundamental biochemical reactions involve the presence of N, which is the fourth most-consumed nutrient of cultivated plants. In the case of some plants in the Leguminosae family, N can be fully or partially provided through the symbiosis of the legume plants with nodulating N_2-fixing bacteria, commonly known as rhizobia.

Rhizobia belong to a particular group of microorganisms that have an enzyme complex called nitrogenase, responsible for the reduction of atmospheric nitrogen (N_2) to ammonia (NH_3). This process is known as Biological Nitrogen Fixation (BNF) and it is an important N source in agricultural systems, consequently reducing the requirements of N fertilization of legume crops. In Brazil, the best example of BNF application is the inoculation of soybean (*Glycine max*. (L.) Merrill) with strains of the genus *Bradyrhizobium*, making N fertilization completely unnecessary and ensuring greater competitiveness of this commodity in the international market (Vargas et al., 1982; Moreira & Siqueira, 2006).

The rhizobia synthesise signaling molecules that are responsible for the nodule development after the stimulation of flavonoids exuded from the roots of

legumes in the soil (Broughton et al., 2000; Shorupska et al., 2006). These signaling molecules, called Nod factors, are lipochitooligosaccharides that have various chemical substitutions. Nod factors are responsible for initiating root hair curling, infection thread formation and activation of cellular division of cortical cells, resulting in the formation of the nodules (Schulze et al., 1998). Within these nodules, the bacteria differentiate into bacteroids that perform the BNF process. In response, the plants provide carbohydrates as carbon and energy source for these bacteria. Nod factors are not the only bacterial signals necessary for the establishment of a successful symbiosis. As in other interactions between bacteria and plants or animals, surface polysaccharides (SPS) are also involved. These molecules act as important signals in symbiotic processes and are present in Gram-negative bacteria as cyclic glucans, lipopolysaccharides (LPS), capsular polysaccharides (KPS) and exopolysaccharides (EPS), (Spaink, 2000; Fraysse et al., 2003; D'Haeze et al., 2004).

The cyclic glucans are usually concentrated in the periplasmic space, where they are important regulatory compounds involved in the osmotic adaptation of bacteria (Breedeveld et al., 1993). The LPS are anchored in the bacterial outer membrane and consist of three parts: lipid A, the core polysaccharide and the O-antigen polysaccharide (Madigan et al., 2004). The KPS are surface polysaccharides that form a cohesive layer adhered to the bacterial cell surface, while EPS refers to polysaccharides with little or no cell association (Fraysse et al., 2003; Lepek & D'Antuono, 2005; Shorupska et al., 2006) (Figure 1).

This review is focused on the EPS and their function in the process of symbiotic BNF and the adaptation of rhizobia to environmental stresses. Brief considerations of the potential industrial applicability of these bacteria in the production of gums and the importance of these compounds in soil aggregation are also presented.

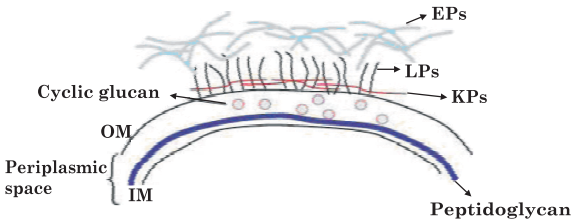

Figure 1. Schematic representation of bacterial surface polysaccharides. EPS: exopolysaccharides; KPS: exopolysaccharides attached to the bacterial surface; LPS: lipopolysaccharides; IM: cell internal membrane; OM: cell outer membrane (Source: Lepek & D'Antuono, 2005).

EXOPOLYSACCHARIDES PRODUCED BY α AND β-RHIZOBIA

Different taxonomic groups of prokaryotes have the capacity of biological N_2 fixation, with high morphological, physiological, genetic and phylogenetic diversity (Moreira & Siqueira, 2006). Until 2001, it was believed that the N_2-fixing bacteria able to form nodules on legume plants were restricted to the α-proteobacteria class, which includes the genera: *Rhizobium* (Frank, 1889), *Ensifer* (*Sinorhizobium*) (Dangeard, 1926; Chen et al., 1988; de Lajudie et al., 1994; Young, 2003), *Allorhizobium* (de Lajudie et al., 1998, Young et al., 2001), *Bradyrhizobium* (Jordan, 1982), *Azorhizobium* (Dreyfus et al., 1988), *Mesorhizobium* (Jarvis et al., 1982, 1997; Jordan, 1984). However, some authors found that bacteria of the genera *Burkholderia* (Moulin et al., 2001) and *Cupriavidus* (*Ralstonia)* (Chen et al., 2001; Vandamme & Conye, 2004), both belonging to the class β-proteobacteria are also able to fix N_2 and form nodules on legumes. Besides, other genera and families in Rhizobiales (α-proteobacteria) were described as N_2-fixing bacteria able to establish symbiosis with legumes: *Devosia* (Rivas et al., 2002, 2003), *Phyllobacterium* (Valverde et al., 2005), *Methylobacterium* (Sy et al., 2001; Jourand et al., 2004), *Ochrobactrum* (Trujillo et al., 2005) and *Shinella* (Lin et al., 2008).

Both α and β proteobacteria are able to produce EPS that can be classified as homo and heteropolysaccharides. Homopolysaccharides are generally neutral glucans, whereas heteropolysaccharides are mostly polyanionic compounds, due to the presence of uronic acid. The EPS produced by rhizobia are mostly species or strain-specific heteropolysaccharides and are formed from repeat units of hexose residues such as glucose, galactose, mannose, rhamnose, and galacturonic and glucuronic acids with pyruvate, acetyl, succinyl and hydroxybutanoic substitutions (Lepek & D'Antuono, 2005). The EPS produced by rhizobia are highly diverse, varying in the type of sugars and their linkage in the single subunit, repeat unit size and polymerization degree, as well as non-carbohydrate decoration (van Workun et al., 1998; Laus et al., 2005; Fraysse et al., 2003; Shorupska et al., 2006). Figure 2 shows the primary structure of EPS from different rhizobia species. Among the genera of known α-rhizobia, the EPS composition has only been characterized for *Rhizobium, Bradyrhizobium*, *Sinorhizobium* and *Azorhizobium* so far.

Strains of *Rhizobium leguminosarum*, despite having different biovars (*trifolii*, *viciae* and *phaseoli*) and nodulating different host plants, have conserved EPS composed of glucose, glucuronic acid and galactose at a ratio of 5:2:1 (Robertsen et al., 1981; O'Neil et al., 1991) (Figure 2a). However, some strains secrete EPS with different sugar contents and chain

lengths. In *R. leguminosarum* bv. *trifolii* 4S (Figure 2b), an EPS subunit is composed of seven sugars, and the galactose molecule is absent in this chain (Amemura et al., 1983). In *Rhizobium leguminosarum* bv. *viciae* 248 (Figure 2C) the EPS subunit has an additional glucuronic acid (Canter-Cremers et al., 1991). Similar to *S. meliloti,* strains of *R. leguminosarum* can produce EPS that differ in molecular weight, i.e., they can produce both low and high molecular weight EPS (Djordevic et al., 1987; Mazur et al., 2003).

The chemical composition of EPS produced by other species of the *Rhizobium* genus has also been described, such as the EPS of *R. tropici* CIAT899^T (Figure 2d) composed of subunits consisting of glucose and galactose sugars at a ratio of 6:2 (Gil-Serrano et al., 1990) and the EPS produced by *Rhizobium sullae* strain KYGT207, which is formed from monomers of glucose, galactose and mannuronic acid at a ratio of 2:1:1 (Kaci et al., 2005). *Rhizobium huakuii* isolated from nodules of *Astragalus sinicus* produces EPS composed of glucose, galactose, ribose and glucuronic acid at a ratio of 5:1:1:1 (Hisamatsu et al., 1997) and *Rhizobium* sp. N613 produces a homogenous β-glucan which consists of β-D-(1-4)-glucose and β-D-(1-6)-glucose at a ratio of 2:1 (Zhao et al., 2010).

In *Bradyrhizhobium japonicum*, differences in the composition of EPS (Table 1), DNA sequence, membrane lipid composition and antibiotic resistance led to the reclassification of this species into two groups (I and II). One group continued as *B. japonicum* (group I) while the other was renamed *B. elkanii* (group II) (Kuykendall et al., 1992). The EPS of *B. japonicum* (Figure 2e) is composed of mannose, galactose, glucose, and galacturonic acid sugars at a ratio of 1:1:2:1 (Mort & Bauer 1980, 1982; Huber et al., 1984; Puvanesarajah et al., 1987; Louch & Miller, 2001), while *B. elkanii* (formerly referred to as *B. japonicum* group II) synthesises an EPS consisting solely of rhamnose and glucuronic acid at a ratio of 3:1 (Huber et al., 1984; An et al., 1995) (Figure 2f).

Among the best-known EPS produced by rhizobia is succinoglycan (EPS I), produced by strains of *Sinorhizobium meliloti* (Figure 2g). This EPS is composed of an octasaccharide of repeat units containing one galactose and seven glucoses (Leigh et al., 1985; Reinhold et al., 1994). *S. meliloti* also has the ability to synthesise another exopolysaccharide, galactoglucan (EPS II), which is synthesized under low-phosphate conditions or when mutations in genes related to EPS I synthesis occur (Zhan et al., 1989; Zhan et al., 1991; Keller et al., 1995). EPS II is a disaccharide with repeat units composed of one glucose and one galactose (Zhan et al., 1989; Her et al., 1990) (Figure 2h). Both EPS I and II may be secreted in two different fractions, high or low molecular weight. The high molecular weight fractions of EPS I and II have hundreds to thousands of monomeric units (10^6–10^7 Da), while the low-molecular-weight fractions consist of monomers, dimers and trimers in the case of EPS I and oligomers (15 to 20 units) in the case of EPS II (Gonzalez et al., 1996; Gonzalez et al., 1998; Wang et al., 1999; Shorupska et al., 2006).

The EPS produced by *Azorhizobium caulinodans* strain ORS571^T, different from other EPS produced by species of rhizobia, is a linear homosaccharide

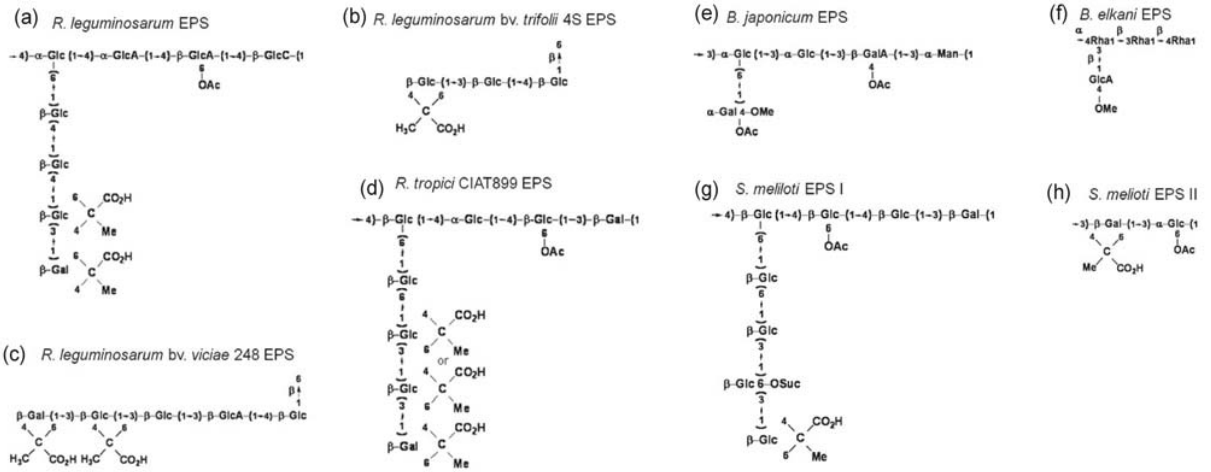

Figure 2. Primary EPS structure of different rhizobia species (a) *Rhizobium leguminosarum* (Robertsen et al., 1981; O'Neil et al., 1991), (b) *R. leguminosarum* bv. *trifolii* 4S (Amemura et al., 1983), (c) *Rhizobium leguminosarum* bv. *viciae* 248 (Canter-Cremers et al., 1991), (d) *Rhizobium tropici* CIAT899^T (Gil-Serrano et al., 1990), (e) *Bradyrhizobium japonicum* (Mort & Bauer 1980, 1982; Huber et al., 1984; Puvanesarajah et al., 1987; Louch & Miller, 2001), (f) *Bradyrhizobium elkani* (Huber et al., 1984; An et al., 1995), (g) *Sinorhizobium meliloti* EPS I (Leigh et al., 1985; Reinhold et al., 1994), (h) *Shinorhizobium meliloti* EPS II (Zhan et al., 1989; Her et al., 1990). Glc: glucose; Gal: galactose; GlcA: glucouronic acid; GalA: galactouronic acid; Mn: manose; Rha: rhamnose; Suc: succinate; Ac: acetate.

Table 1. Composition of EPS in repeat units normalized to mannose, from the different *Bradyrhizhobium japonicum* strains previously classified as group I and group II. (Modified and updated from Huber et al., 1984) (Note: Currently group II is classified as *B. elkanii*)

Group/Strain	Mannose	Glucose	Galactouronic acid	Galactose	4-*O*-Methyl galactose	Rhamnose	4-*O*-Methyl-galacturonic acid
I							
ATCC10324[(1)]*	1	1.54	0.87	0.57	0.25	-***	-
D193[(1)]	1	1.64	0.68	0.59	0.29	-	-
D209[(1)]	1	0.83	0.46	0.38	0.09	-	-
HS123[(3)]	1	2	-	0.6	0.4	-	-
M1E7[(4)]	1	2	0.81	0.81	-	-	-
THA6[(1)]	1	1.59	0.93	0.60	0.25	-	-
USDA 24[(1)]	1	1.59	0.91	0.60	0.25	-	-
USDA 38[(1)]	1	1.85	0.99	0.74	0.19	-	-
USDA 58[(1)]	1	2.03	0.52	0.73	0.15	-	-
USDA 62[(1)]	1	1.83	0.60	0.66	0.41	-	-
USDA 110[(1)]	1	2.20	1.10	0.52	0.42	-	-
USDA 115[(1)]	1	1.65	0.84	0.62	0.23	-	-
USDA 123[(1)]	1	2.17	0.71	0.35	0.70	-	-
USDA 140[(1)]	1	1.96	0.97	0.75	0.27	-	-
3I1b 138[(2)]	1	2.30	1.18	0.32	0.68	-	-
3I1b 110[(2)]	1	2.20	1.12	0.52	0.42	-	-
61A50[(1)]	1	1.75	0.75	0.50	0.32	-	-
5631[(1)]	1	1.74	0.88	0.76	0.27	-	-
5633[(1)]	1	1.58	0.79	0.45	0.39	-	-
II							
USDA 29[(1)]	-	-	-	-	-	3	-
USDA 46[(1)]	-	-	-	-	-	3	1
USDA 76[(1)]**	-	-	-	-	-	3	+****
USDA 86[(1)]	0.36	-	-	-	-	3	+
USDA 94[(1)]	0.25	0.10	-	-	-	3	+
USDA 117[(1)]	-	-	-	-	-	3	+
USDA 130[(1)]	0.19	0.34	-	-	-	3	+
61A76[(1)]	-	-	-	-	-	3	+

[(1)] Huber et al. (1984). [(2)] Mort & Bauer (1980). [(3)] Puvanesarajah et al. (1987). [(4)] Louch & Miller (2001). Type strains of *: *B.japonicum* and **: *B.elkanii*; ***: absence of monosaccharide; ****: Presence of monosaccharide, at an undetermined level; Adapted from Huber et al. (1984).

composed only of 4,6-*0*-(1-carboxyetilideno)-D-galactosyl residues (D' Haeze et al., 2004).

The β-rhizobia genus *Burkholderia* contains both associative and symbiotic species. The EPS structure of the N_2-fixing strains of the symbiotic species *B. caribensis* MWAP71 is composed of glucose and thalose in a 2:1 ratio (Vanhaverbeke et al., 2001). As an example of the EPS described for associative species of N_2–fixing *Burkholderia,* we can cite the *B. kuruensis* strain M130, which produces two distinct EPS, EPS A and EPS B. EPS A is composed of rhamnose, glucose and glucuronic acid at a ratio of 2:2:1, whereas EPS B is a mixture of two polymers of hepta or octasaccharide repeat units composed of rhamnose, galactose, glucuronic acid and glucose at a ratio of 2:2:2:1 or 2:2:2:2 (Mattos et al., 2001; Hallack et al., 2010). *B. tropica* Ppe8 is not actually a valid species, but a study of the composition of its EPS revealed that it is formed by subunits composed of rhamnose, glucose and glucuronic acid at a ratio of 2:2:1 (Serrato et al., 2008).

A wide variety of chemical structures of different rhizobia species were described, however, the chemical composition of the EPS of some genera such as *Mesorhizobium* have not been determined yet. The chemical characterization of the EPS of other rhizobia species is still needed, since the characterization of these compounds is highly relevant from an economic and agricultural point of view. It should also be emphasized that these studies dealt mainly with strains from temperate regions, which are adapted to quite different weather and soil conditions from those in tropical regions.

FUNCTION OF EXOPOLYSACCHARIDES IN THE PROCESS OF LEGUME NODULATION

The main steps for the establishment of the symbiosis between rhizobia and legume species are: rhizobia multiplication on root surface, rhizobia

adhesion to root surface, root hair curling, infection thread formation (in root hairs), formation of nodule meristem, nodule development and differentiation, release of rhizobia from infection threads, their division and differentiation into bacteroids, development of nitrogenase, biochemical and physiological functions associated with N_2 fixation and maintenance of nodule function (Sprent, 1989).

The biological functions of EPS in rhizobium-legume symbiosis are related to different stages of plant infection by the bacterium. It has already been shown that these compounds are essential for the effective establishment of the symbiosis between *Rhizobium* sp. NGR234 and *Leucaena leucocephala* or *Macroptilium atropurpureum*. Mutant strains deficient in EPS production were unable to promote the formation of efficient nodules on these different hosts, and the ability to induce functional nodules with these mutants was restored by adding purified EPS from the parental strain (Djordjevic et al., 1987). EPS production is also indispensable in the nodule formation process by *M. tianshanense,* and no nodules were formed when EPS mutant strains were inoculated on roots of *Glycyrrhiza uralensis* (Wang et al., 2008).

A mutation in *R. leguminosarum* bv. *trifolii* 24.1 for EPS production (*exo*$^-$ mutants) formed inefficient nodules in *Trifolium pratense* plants (Skorupska et al., 1995), and the EPS of *R. leguminosarum* bv. *viciae* ANU843 was necessary to induce root hair curling and infection thread formation in *V. sativa* (van Workun et al., 1998). Inoculation with *exo*$^-$ mutants of *R. leguminosarum* RBL5523 on the roots of *V. sativa* subsp. *nigra* also blocked the infection thread formation, which was aborted soon after initiation of the infection process (Laus et al., 2004, 2005). The co-inoculation of mutant *exo*$^-$ Nod$^+$ mutants with *exo*$^+$ Nod$^-$ mutants restored the nodule development process in *Rhizobium* bacteria, demonstrating the importance of EPS in the nodulation process (van Workun et al., 1998; Laus et al., 2005).

The influence of EPS on root hair curling and bacteria invasion into the nodule was assessed with different strains mutated for EPS production (*exo*$^-$), using *S. meliloti* Rm1021 symbiosis with *Medicago sativa* plants. Strains with the *exo*$^-$ phenotype were not capable of nodulating these plants efficiently (Leigh et al., 1985; Battisti et al., 1992; Urzainqui & Walker, 1992). The addition of small EPS quantities produced by wild strains during inoculation with *exo*$^-$ mutants allowed the formation of functional nodules. The addition of low-molecular weight EPS I (succinoglycan) at the moment of inoculation of *exo*$^-$ mutants of *S. meliloti* Rm1021 led to the formation of a nodule morphology similar to the wild type strain, with the presence of a large quantity of bacteroids (Battisti et al., 1992; Urzainqui & Walker, 1992).

A mutant of *S. meliloti* Rm2011 unable to produce EPS I only induced the formation of pseudonodules that did not contain an infection thread or bacteroids in *M. sativa* (Niehaus et al., 1993). An intact structure of the EPS I of *S. meliloti* Rm1021 is required for the initial infection thread formation and elongation (Cheng & Walker, 1998), suggesting that the EPS functions as a signaling molecule that recognizes complex receptors present in plants. A EPS I mutant of *S. meliloti* CXM1-118 inoculated into *M. sativa* and *M. trunculata* plants formed small, irregularly shaped and inefficient nodules that were formed three to four days after those induced by the parental strain (Zatovskaya et al., 2007). Electron microscopy analysis of these nodules revealed the presence of highly vacuolated cells that contained a reduced number of bacteroids.

In the case of *S. meliloti* Rm1021, EPS II is also involved in the formation of nodules that fix N_2 efficiently, overcoming the symbiotic defects of strains deficient in the production of EPS I, which are able to functionally replace succinoglycan (Glazebrook & Walker, 1989; Gonzalez et al., 1996). Moreover, the addition of low-molecular-weight EPS II to mutants deficient in the production of EPS I and II enabled the process of N_2 fixation, producing nodules morphologically indistinguishable from those produced by the wild type strain (Gonzalez et al., 1996). Also, the symbiotically active fraction of EPS II (low-molecular-weight fraction) is shown to be a critical factor for biofilm formation and root colonization. Thus, the ability of *S. meliloti* Rm1021 to properly attach to root surfaces and form biofilms conferred by the synthesis of EPS may embody the main function of these symbiotically essential molecules (Rinaudi & González, 2009).

The EPS also play a fundamental role in the initial stages of the symbiotic interaction between *Bradyrhizobium japonicum* 110spc4 and *Glycine max* in preventing the defense response of the host plant. EPS mutant strains (*exo*$^-$) of *B. japonicum* stimulated the accumulation of phytoalexins (defense substances produced by plants) in the early stages of interaction with *Glycine max.* After 72 h of incubation, the levels of phytoalexins produced by the plants were 10-fold higher than in plants inoculated with the wild type strain (Parniske et al., 1993, 1994).

Exo$^-$ mutants of *S. meliloti* Rm2011, when inoculated on *M. sativa*, formed pseudonodules that induced an alteration in the defense response of the plants, with increased wall thickness of the cortical cells and an accumulation of phenolic compounds in the cells of these nodules (Niehaus et al., 1993). In the symbiosis of *R. leguminosarum* bv. *trifolli* 24.1 with *T. pratense*, EPS-deficient mutants induced the accumulation of phenolic compounds and necrosis in the cortical cells of the host plant, which indicates a plant defense reaction in response to the infection process (Shorupska et al., 1995). In mutants that produced small amounts of EPS, the defense reaction of the plant was not as strong; infection threads were formed, but resulted in the development of irregularly shaped bacteroids and an electron-dense cytoplasm, which are both signs of degeneration (Bialek et al., 1995).

In the *Azorhizobium caulinodans* ORS571^T - *Sesbania rostrata* symbiosis, mutants deficient in EPS production were unable to penetrate the tissues of the host plant due to the loss of protection by EPS upon exposure to H_2O_2, produced by *Sesbania rostrata* as a defense mechanism (D' Haeze et al., 2004).

The function of EPS in the determination of rhizobia-host plant specificity is still very controversial (Shorupska et al., 2006). Van Workun et al. (1998) demonstrated that *exo*$^-$ mutants of *R. leguminosarum* bv. *viciae* RBL5523 inoculated into *V. sativa* can overcome the lack of EPS production when homologous EPS (structurally similar), but not heterologous EPS (structurally different) are added, suggesting that there are some structural requirements for EPS to function as a signaling molecule in the process of symbiosis. A hybrid strain of *Rhizobium* sp NGR234 containing the genes of *S. meliloti* for the production of EPS I was capable of inducing the formation of nodules in *L. leucocephala* plants, which however were not able to fix N_2, indicating that the EPS structure is essential for the formation of efficient nodules (Gray et al., 1991).

In co-inoculation experiments using the RBL5833 *exo*$^-$ strain of *R. leguminosarum* with other species of EPS-producing bacteria (*Agrobacterium tumefaciens* LBA4301, *Rhizobium* NGR234, *R. leguminosarum* bv. *trifolii* ANU845 and LPR5045, *R. tropici* CIAT899^T, *S. meliloti* RCR2011) in *V. sativa* roots, nodules were formed only on roots inoculated with rhizobia producing EPS homologs (*R. leguminosarum* bv. *trifolii* ANU845 and LPR5045) and with rhizobia producing similar EPS (*R. tropici* CIAT899^T). However, the transformation of heterologous strains with the symbiotic pRL1J1 Sym plasmid of *R. leguminosarum* enabled these strains to form efficient nodules in *V. sativa* plants, which indicates that the specificity of N_2-fixing bacteria is not determined exclusively by the EPS structure (Laus et al., 2005).

Considering that a limited number of rhizobia species deficient in EPS production were investigated for co-inoculation with non-homologous EPS-producing species, the hypothesis that EPS are involved in specificity to the host plant cannot be confirmed. Therefore, it is not possible to relate EPS with specificity of N_2-fixing bacteria and legumes.

EXOPOLYSACCHARIDES IN THE PROCESS OF RHIZOBIA ADAPTATION TO LIMITING ENVIRONMENTAL CONDITIONS

For the establishment of the symbiosis between rhizobia and legumes, in addition to the requirements for recognition of specific chemical signals between the symbionts, the environmental conditions must be adequate for the development of this interaction. In tropical regions it is common to find highly acidic soils associated with toxic Al, salinity, low levels of Ca and P, high temperatures, and other types of stresses. Most arable soils have low pH (< 5.0) contributing to reduction in nutrient availability such as Ca^{2+} and Mg^{2+} and increasing the concentrations of toxic elements such as Al^{3+} and Mn^{2+} (Ribeiro et al., 1999). Phosphorus rarely exceeds 5.0 mg dm^{-3}, generally ranging from 1.2 to 1.5 mg dm^{-3} (Mello et al., 1983). According to the classes of interpretation of P availability, these levels are very low (Ribeiro et al., 1999). Moreover, the soil surface layer can reach temperatures of around 40 °C (Hafeez et al., 1991). Soils with these characteristics may not only limit plant growth, but the survival of rhizobia in the soil, its infection of the plant and the process of BNF as well.

As described above, the EPS produced by rhizobia are very diverse in composition and chemical structure. Besides, under normal cultivation conditions, there is great variability in the production of EPS by rhizobia strains, both quantitatively and qualitatively. The strains with high levels of EPS production tend to be more tolerant to acidic conditions and salinity than strains that produce low EPS levels (Cunningham & Munns, 1984; Eaglesham et al., 1987; Xavier et al., 1998; Freitas et al., 2007; Xavier et al., 2007). In the case of saline stress, the EPS surrounds the bacterial cells, decreasing the cell surface contact with the saline medium and increasing cell resistance to the osmotic effect (Elsheikh & Wood, 1990).

The strains BR 29 and SEMIA 587 of *Bradyrhizobium elkanii*, recommended as soybean inoculants, produce greater amount of EPS when grown in acidic conditions than when cultivated at a neutral pH (Barberi et al. 2004; Miguel & Moreira, 2001). The same trend of increased production of EPS under acidic conditions and with limited Ca^{2+} was observed in the strain USDA 3187 of *Bradyrhizobium* sp. (Macció et al., 2002).

The limitation of some nutrients, e.g., Ca and P, can also lead to increased EPS production by certain rhizobia strains. In these cases, the increased EPS production is considered an adaptation mechanism of these bacteria (Barberi et al., 2004; Macció et al., 2002). Thus, the EPS synthesis by rhizobia can also be regulated by the culture conditions.

As previously mentioned, *Sinorhizobium meliloti* produces two types of EPS, and the concentration of phosphate in the medium regulates the production of one type of EPS at the expense of the other. Under low-phosphate conditions EPS II predominates, and the colonies of these bacteria have a more mucoid morphology. Under normal conditions, *S. meliloti* produces EPS I, with less mucoid colonies (Zhan et al., 1991; Mendrygal & Gonzalez, 2000).

However, abiotic stresses do not always induce greater production of EPS by rhizobia strains. This was observed for *S. meliloti* strains grown under acidic conditions at low Ca^{2+} concentrations. Under these conditions, the limitation of Ca^{2+} in the culture medium drastically reduced the production of EPS by the strains (Dilworth et al., 1999; Delavechia et al., 2003).

The different rhizobium genera differ in EPS production when the strains are cultivated under different environmental conditions, causing alterations in the production and chemical composition of these compounds. The species or strain-specific EPS substances are essential for the establishment and effectiveness of the symbiosis between rhizobia and legumes, and it is important to understand the influence of these characteristics on the nodulation process.

Studies on EPS I and II produced by *S. meliloti* were performed in order to determine the function of these two compounds during nodulation of *M. sativa*. It was observed that EPS I is more efficient in mediating the invasion processes, although both EPS act in the process of *M. sativa* nodulation (Pellock et al., 2000). These authors report that the ability of *S. meliloti* to produce two types of EPS and their nodulation process represents a competitive advantage of this strain, since even under limiting environmental conditions the process of nodulation and N_2 fixation is not affected.

Due to their predominantly anionic nature, the EPS have the capacity to strongly interact with metal cations and play an important role in the sequestration or immobilization of these ions in the environment (De Philippis & Vincenzini, 1998). Despite the increase in EPS production in response to heavy metals studied in other bacterial species, few studies on rhizobia have been performed (Santamaría et al., 2003). EPS produced by *Bradyrhizobium* (Chamaecytisus) BGA-1 and *Bradyrhizobium japonicum* USDA 110 in the presence of solutions of Fe^{3+}, Al^{3+} and Th^{4+} form a gelatinous precipitate composed of EPS bound to these metals (Corzo et al., 1994; Santamaría et al., 2003; Diaz-Marrero et al., 2004). The EPS of *Rhizobium etli* is also able to bind to metal ions, and is able to rapidly adhere to Mn^{2+} and Pb^{2+} (Foster et al., 2000). The complexation of Cd^{2+} by the bacterium *S. meliloti* can be also the result of the attachment of this ion to extracellular polymeric substances and the amount of Cd^{2+} bound to the EPS increases at high Cd^{2+} concentrations (Slaveykova et al., 2010), suggesting a potential application of this biopolymer in the field of bioremediation.

POSSIBLE INDUSTRIAL APPLICATIONS OF RHIZOBIUM EPS

EPS produced by some microorganism species are widely used in various industrial activities. These compounds, also called biopolymers, are hydrosoluble gums with the ability to form gels and viscous solutions in an aqueous medium.

Microbial biopolymers vary greatly in their composition and consequently in their physical and chemical properties. Due to this wide diversity in both structure and physical properties (high viscosity, networks of intermolecular cohesive properties) the applications of these compounds is broad in the food, pharmaceutical, petroleum, cosmetic, textile, paint industry and agricultural products (Bryers, 1993).

Dextran, xanthan and gellan produced by the bacteria *Leuconostoc* spp., *Xanthomonas* spp. and *Sphingmonas elodea* respectively, are still among the few microbial polysaccharides marketed on a large scale, and these compounds are very important in the gum market. The structure of these polysaccharides is rather varied. Xanthan is composed of glucose, mannose and glucuronic acid at a ratio of 2:2:1. Dextran is a homopolysaccharide composed of glucose molecules, while gellan is a heteropolysaccharide consisting of glucuronic acid, glucose and rhamnose with glycerate and acetate groups in its structure. Economically, xanthan is the most important microbial polysaccharide, with a worldwide production of about 40 to 50 thousand tons/year and a value of about 270 million U.S. dollars annually. The annual demand is estimated to increase at a continuous rate of 5 to 10 % (Pradella, 2006). Table 2 shows the main examples of bacterial EPS applied in various industrial activities.

It is economically attractive to search for new polysaccharide-producing microorganisms. The production of large quantities is a challenge being met by several research groups, as microbial biopolymers can be produced in large quantities and fermentation offers the advantage of controlled production. This eliminates the problems found in the production of polymers by plants and algae, e.g., problems with harvesting, climate conditions or marine pollution (Sutherland, 2001).

The atmospheric N_2 fixing bacteria (NFB) can be found as both free-living organisms and in association or symbiosis (e.g. rhizobium-legumes). Studies on commercial EPS applications are more advanced for the free-living NFB, e.g., clairana produced by *Beijerinckia* sp. (Moreira et al., 2003) and the alginate produced by *Azotobacter vinelandii* (Garcia-Cruz et al., 2008).

Since there are no studies on the commercial production of gum by rhizobia, these can be considered unexplored sources of microbial polysaccharides, highly promising for industrial applications. These bacteria have high morphological, physiological, genetic and phylogenetic diversity, which can be a valuable source for the screening of strains with target properties. Furthermore, they are not pathogenic and produce large amounts of EPS. In figure 3 we illustrate the appearance of colonies characterized by

Table 2. Exopolysaccharides produced by bacteria (Adapted from Bryers, 1993)

EPS	Microorganism	Main use
Alginate	*Azotobacter vinelandii*	Gelling agent
Curdlan	*Alcaligenes faecalis*	Paint thickener
Dextran	*Leuconostoc Mesenteroides, Klebsiella* spp.	Viscosity modifiers, photographic industry, dietary sugar
Gellan	*Sphingomonas paucimobilis (syn.: Pseudomonas elodea)*	Texturizing, stabilizer, thickener, emulsifier and gelling agent
Marinactan	*Flavobacterium uliginosum*	Anticancer and antitumor therapy
Xanthan	*Xanthomonas campestris*	Sauces and syrups, toothpaste, bread, cosmetics, agricultural products, paints
Zanflo	*Erwinia tahitica*	Clay stabilizer for drilling petroleum wells

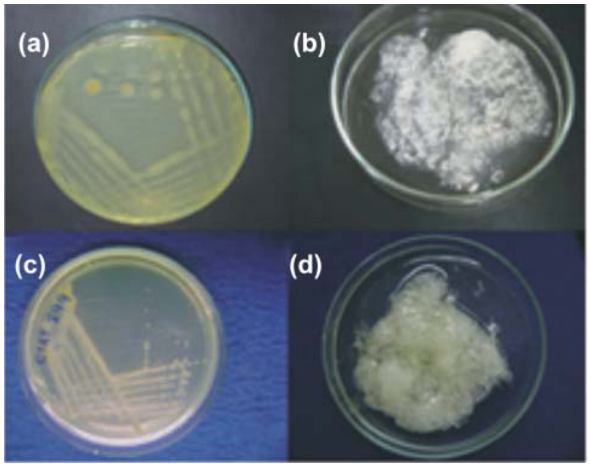

Figure 3. (a) Colonies of the *M. plurifarium* BR3804 strain, grown in 79 medium with bromothymol blue; (b) the biopolymer produced by *M. plurifarium* BR3804 after precipitation with ethanol (1.44 g dry weight EPS/L of medium) after 72 h of culture; (c) colonies of *R. tropici* CIAT899^T strain, grown in 79 medium with bromothymol blue; (d) biopolymer produced by *R. tropici* CIAT899^T after precipitation with ethanol (3.0 g dry weight EPS/L of medium) after 72 h of culture.

high gum production on solid medium, and the precipitation of EPS from strains of *Mesorhizobium plurifarium* BR3804 and *R. tropici* CIAT899^T, recommended by MAPA (Ministério da Agricultura, Pecuária e Abastecimento) as inoculants for the species *Chamaecrista ensiformis* and *Phaseolus vulgaris,* respectively, demonstrating the high potential of these strains for the production of these biopolymers.

ROLE OF EPS IN SOIL AGGREGATION

Soil aggregation is essentially related to suitable physical conditions underlying plant growth. The aggregation process is related to several factors, such as the physical properties, climatic conditions and biological activities in the soil (Materechera et al., 1994; Bezzate et al., 2000).

The action of microorganisms, notably fungi and bacteria, in the process of soil particle aggregation is principally related to the EPS production by these organisms (Alami et al., 2000). Microbial EPS can increase the adhesion of soil particles to plant roots and the mechanical stability of rhizospherical soils, besides increasing the level of water retention in this environment (Chenu & Roberson, 1996; Amellal et al., 1998).

The inoculation of wheat (*Triticum durum* L.) with the associative bacterium *Paenibacillus polymyxa*, selected by its ability to fix N_2, resulted in a 57 % increase of the soil mass that adhered to the roots and an increased frequency of aggregates with sizes between 0.2 and 2 mm due to EPS production (levan), which contributed to the aggregation of this soil (Gouzou et al., 1993; Bezzate et al., 2000). The same effect on wheat was also observed after inoculation with other associative species. Inoculation of wheat seedlings in soil with 24 % water content with *Pantoea aglomerans* resulted in an increase in the adhesion of soil particles to the roots: 140 mg of soil adhered to 1 mg of roots in the inoculated treatment compared to 90 mg of soil that adhered in the control treatment. There was also a significant increase in the macroporosity of the inoculated soil (diameters of 10 to 30 μm) compared to the uninoculated control (Amellal et al., 1998).

The effect of inoculation of clay soils with the diazotrophic cyanobacterium *Nostoc* spp. resulted in an increased incidence of porosity in the inoculated soils (30 %) compared with the control soil without inoculation (5 %). Electron microscopy analysis allowed the visualization of the primary soil aggregation that resulted from the interaction of the secreted EPS with soil particles (Falchini et al., 1996). These cyanobacteria can establish symbiosis with species of fungi, bryophytes, gymnosperms, and

angiosperms or live freely in the soil. The EPS of *Nostoc* ssp. contributes not only to the soil biochemical properties but also to soil fertility, once the EPS produced by this cyanobacterium increases the soil C pool as carbohydrates (Maqubela et al., 2009). Also, the water retained in the EPS matrix reduces evaporation losses and potentially increases the water-holding capacity of the soil (Mager, 2010).

Some studies relating the production of EPS by strains of rhizobia and soil aggregation have also been described. Inoculation of a *Rhizobium* sp. strain into *Triticum durum* L. plants significantly increased the percentage of aggregates with a diameter of 1.6 to 2 mm, and the water stability in this fraction was 42 ± 5 % in the inoculated treatments compared with 30 ± 4 % in the control (Kaci et al., 2005). Another strain of *Rhizobium* sp., promoting growth of *Helianthus annuus* L. provided a significant increase in the volume of macro pores (diameter from 12 to 60 μm) in soil inoculated with this bacterium. Under water stress, the soil structure around the root system changed, which avoided negative effects of water deficit on the growth of the inoculated plants (Alami et al., 2000). In another study it was observed that EPS synthesis in *Rhizobium* sp. YAS34 is also decisive for the colonization of the basal part of the root system and that it increases the stability of root-adhering soil on *Arabidopsis thaliana* and *Brassica napus* roots (Santaella et al., 2008).

Conformational studies of the structure of EPS from *Burkholderia caribensis* MWAP71 demonstrated that this strain produces an EPS responsible for aggregation by the adsorption capacity to mineral surfaces and the adhesive properties of this biopolymer (Vanhaverbeke et al., 2003).

Although little attention has been paid to the influence of microorganisms in the process of soil aggregation, particularly to bacteria-producing EPS, microbial EPS are important biological factors that influence the soil structure formation. These compounds contribute to the stability and aggregation of particles and are potential agents for improving the structural quality of agricultural soil. Thus, the magnitude of such effects should be quantified, to evaluate the feasibility of its management for improving soil physical conditions.

FINAL CONSIDERATIONS

The BNF process based on the legume-*Rhizobium* symbiosis leads to a high sustainability of agricultural systems. BNF increases soil fertility and organic matter levels and reduces the need for N fertilization, resulting in both economic and environmental benefits. Despite all data available in the literature about the possible roles of EPS in the symbiosis establishment and functioning, the exact role of these compounds in the process is not yet completely understood, and further studies on the signaling mechanisms is required. Previous research of the role of EPS in nodulation and BNF based on the study of mutant strains for EPS production was focused mainly on the genera *Rhizobium* and *Sinorhizobium*, while the understanding of these processes in other species is scarce or absent. Thus, research and comparisons among the wide range of other genera are necessary.

Although there is evidence of strain- and species-specificity, EPS production by rhizobia is regulated by environmental conditions which, in some cases, increases the ability to adapt to various stress conditions. Thus, studies related to the role of EPS in the process of adaptation of these bacteria to various edaphic and climate conditions are particularly important, since EPS affect the survival and functionality of the rhizobia strains.

In recent years, the industrial demand for bacterial EPS (biopolymers) has grown significantly. These compounds are widely applicable in various fields, due to their diverse structural and physico-chemical properties. Bacterial EPS offer several advantages, such as the potential for controlled, high-speed production with higher yield and greater purity and consistency than alternative sources. Nevertheless, information on rhizobial EPS is not available, representing a wide range of unexploited possibilities.

EPS also offer a potencial application in agriculture due to it adhesive properties and it ability to form gels that promote the adhesion of soil particles, forming stable aggregates that contribute to better plant growth and development. Although the role of bacterial EPS in soil aggregation is recognized, little information is available in the literature about the specific action of known bacteria and possible methods of management.

Considering that none of the α and β-rhizobia were shown to be pathogenic so far, they can be generally characterized as an unexplored source of microbial EPS with great potential in industrial applications and as stabilizing soil agents. Furthermore, the role of these compounds in stress adaptation may be an important criterion for the selection of inoculant strains to raise plant productivity by BNF under different soil and climatic conditions. The biodiversity of rhizobia in tropical soils represents a vast and unexplored field calling for research in this area.

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