

Evaluation of the biotechnological potential of *Rhizobium tropici* strains for exopolysaccharide production



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ABSTRACT

Rhizobium tropici, a member of the Rhizobiaceae family, has the ability to synthesize and secrete extracellular polysaccharides (EPS). Rhizobial EPS have attracted much attention from the scientific and industrial communities. Rhizobial isolates and *R. tropici* mutants that produced higher levels of EPS than the wild-type strain SEMIA4080 were used in the present study. The results suggested a heteropolymer structure for these EPS composed by glucose and galactose as prevailing monomer unit. All EPS samples exhibited a typical non-Newtonian and pseudoplastic fluid flow, and the aqueous solutions apparent viscosities increased in a concentration-dependent manner. These results serve as a foundation for further studies aimed at enhancing interest in the application of the MUTZC3, JAB1 and JAB6 strains with high EPS production and viscosity can be exploited for the large-scale commercial production of Rhizobial polysaccharides.

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1. Introduction

Many species of bacteria possess the ability to synthesize and excrete extracellular polysaccharides (exopolysaccharides, EPS). Once transported to the extracellular space, EPS exist as either soluble or insoluble polymers and are either loosely attached to the cell surface or completely excreted into the environment as slime. It has been shown that bacterial EPS provide protection from various environmental stresses, such as desiccation, predation, and the effects of antibiotics (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). However, the interest in EPS has increased considerably in recent years because these compounds are candidates for many

commercial applications in the health, bionanotechnology, food, cosmetics, and environmental sectors.

Several researchers have discussed recent advancements in the understanding of the potential industrial applicability of these bacteria for the production of gums and the importance of these compounds in soil aggregation. In addition, the functional properties of bacterial exopolysaccharides have been demonstrated in a wide range of applications, including food products, pharmaceuticals, bioemulsifiers (Xie, Hao, Mohamad, Liang, & Wei, 2013), bioflocculants (Sathiyarayanan, Kiran, & Selvin, 2013), chemical products (Wang, Ahmed, Feng, Li, & Song, 2008; Shah, Hasan, Hameed, & Ahmed, 2008), the biosorption of heavy metals (Mohamad et al., 2012), and antibiofilm agents (Rendueles, Kaplan, & Ghigo, 2013) in both industry and medicine (Nwodo, Green, & Okoh, 2012; Donot et al., 2012). In the agriculture sector, the fluidity of fungicides, herbicides, and insecticides has been improved by the addition of xanthan, which results in the uniform suspension of solid components in formulations (DeAngelis, 2012). Thus, studies in this area are very important for the identification of both novel biopolysaccharides and new techniques for optimizing their production (Bomfeti et al., 2011).

Numerous types of exopolysaccharides have already been described (Castellane & Lemos, 2007; Monteiro et al., 2012; Mota et al., 2013; Radchenkova et al., 2013; Silvi, Barghini, Aquilanti,

Abbreviations: EPS, exopolysaccharide; CDW, cell dry weight; RP-HPLC, reverse-phase high-performance liquid chromatography; UV-vis, ultraviolet–visible; Glc, glucose; Gal, galactose; GalA, galacturonic acid; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose; PMP, 1-phenyl-3-methyl-5-pyrazolone; EPSWT, exopolysaccharide from *Rhizobium tropici* SEMIA4080; EPSC3, exopolysaccharide from the MUTZC3 mutant strain; EPSPA7, exopolysaccharide from the MUTPA7 mutant strain; EPSJ1, exopolysaccharide from the rhizobial isolate JAB1; EPSJ6, exopolysaccharide from the rhizobial isolate JAB6.

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Juarez-Jimenez, & Fenice, 2013). The extracellular polysaccharide, succinoglycan, is produced by *Sinorhizobium*, *Agrobacterium* and other soil bacteria (Simsek, Mert, Campanella, & Reuhs, 2009). However, considering the biodiversity of the microbial world and the number of papers published each year describing new microbial exopolysaccharides, it is astonishing to realize that only three EPS (i.e., dextran, xanthan, and gellan gums) have been successfully adopted for industrial purposes (Donot et al., 2012; Prajapati, Jani, Zala, & Khutliwala, 2013).

One of the well-known EPS producers are rhizobia, which excrete large amounts of polysaccharides into the rhizosphere and, when grown in pure cultures (Noel, 2009), produce copious amounts of EPS, causing increased viscosity. To date, the synthesis of rhizobial EPS has been best studied in two species, namely *Sinorhizobium meliloti* and *Rhizobium leguminosarum* (Marczak, Dzwierzynska, & Skorupska, 2013). The latest data indicate that EPS synthesis in rhizobia undergoes very complex hierarchical regulation, which includes the participation of proteins engaged in quorum sensing and the regulation of motility genes. Previous reports have shown that biofilm formation and exopolysaccharide production by bacterial strains significantly contribute to soil fertility and improve plant growth (Qurashi & Sabri, 2012). However, at present, the role of EPS in these processes is not well understood. Therefore, there is great interest in elucidating the patterns of gene expression and the biochemical processes involved in the production of bacterial extracellular polysaccharides (Marczak et al., 2013).

Based on its superior characteristics as a common bean (*Phaseolus vulgaris* L.) root-nodule symbiont, strain PRF 81, which is known commercially as SEMIA 4080, is the type-strain of *Rhizobium tropici* that currently recommended (authorized) for the production of commercial rhizobial inoculant for common bean production in Brazil (Hungria et al., 2000). Because of its ability to produce large quantities of polysaccharides, this bacterium may prove to be an excellent model species for the development of biotechnology products. Industrial biopolymer production would occur under the same conditions used for the industrial cultivation of rhizobia for soil inoculation in Brazil. Thus, the production of EPS may represent an alternative for this sector because the market for inoculum production in Brazil is highly dependent on the production of soybean [*Glycine max* (L.) Merr.] and common bean. In Brazil, the inocula is only sold between August and December, which is the planting period for the entire country.

To date, there is little information on the physicochemical properties and rheological properties of purified EPS from *R. tropici* strains or on their use in various industrial applications. The *R. tropici* strain SEMIA 4080 combines the advantages of non-pathogenicity and rapid productivity and hence proved to be a very promising model organism and cell factory for microbial EPS production. However, this study is useful for the bio-inoculant producing industries in Brazil as best alternative activity during the non crop season of the common bean and soybean. This study investigated the rheological properties of the EPS from wild-type strain of *R. tropici* SEMIA 4080, mutant strains (MUTZC3 and MUTPA7) and rhizobial isolates (JAB1 and JAB6) to discover its potential as a soil-stabilizing agent or as a rheological modifier of aqueous systems.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The wild-type strain of *R. tropici* SEMIA 4080, mutant strains (MUTZC3 and MUTPA7) and rhizobial isolates (JAB1 and JAB6) were used in the present study. A Rhizobial strain designated JAB1 was

isolated from the common bean (*Phaseolus vulgaris* L.) and classified as *R. tropici*, while the rhizobial isolated designated JAB6 was isolated from pinto peanut (*Arachis pintoi*) and classified as *Rhizobium* sp. For routine *Rhizobium* growth, tryptone yeast (TY) medium (Beringer, 1974) was used. When required, the media was supplemented with the antibiotic kanamycin. The mutants were cultivated in YMA medium (0.4 g L⁻¹ yeast extract, 10 g L⁻¹ mannitol, 0.5 g L⁻¹ K₂HPO₄, 0.2 g L⁻¹ MgSO₄, and 0.1 g L⁻¹ NaCl, 9 g L⁻¹ agar, pH 7.0) (Vincent, 1970) supplemented with Congo Red (25 µg mL⁻¹) to verify the purity of each mutant culture. The cultures were incubated at 30 °C for 24 h.

For comparative analyses of the EPS production obtained with the wild-type, mutant strains of *R. tropici* and rhizobial isolates, the monosaccharide compositions, and the rheological properties of their EPS, pre-inocula and batch experiments were performed using PGYA, PGYL, PSYA, and PSYL media. The detailed contents of these cultivation media are not available because the formulas are under patent restriction (registration PI0304053-4).

2.2. EPS detection and production

For phenotypic comparisons, experiments were conducted using Petri dishes containing solid PSYA medium, sucrose (30 g L⁻¹), as a carbon source, with and without a fluorescent brightener 28 (calcofluor white; Sigma-Aldrich) with an emission wavelength of 430 nm at a final concentration of 200 µg mL⁻¹. This fluorescent pigment is specific to polysaccharides that contain β-1 → 4 or β-1 → 3 linkages (Wood, 1980). The EPS production by each strain was observed by illuminating the dishes with UV light at 365 nm. The mucoidy of the colonies was determined visually.

For the evaluation of EPS production, pre-inocula were initially prepared from cultures cultivated on solid PGYA medium containing glycerol (10 g L⁻¹), as a carbon source. After 24 h, each inoculating strain was cultivated in 125-mL flasks (20 mL of medium in each) containing PGYL liquid medium on a rotary shaker at 140 rpm for 30 h, at which time a suspension with an optical density at 600 nm (*OD*₆₀₀) of 0.3 was obtained. The temperature was maintained at 30 °C. Aliquots of the corresponding cultures were transferred to 1000-mL Erlenmeyer flasks containing 500 mL of half-liquid PSYL at a final concentration of 0.10% (v/v) and incubated for 144 h at 140 rpm and 29 °C.

2.3. Cell biomass determination

The growth was measured based on the dry weight per volume of the culture. The cell dry weight (CDW) was determined by centrifugation (10,000 × g, 4 °C, 50 min) followed by drying to a constant weight in an oven at 60 °C overnight.

2.4. EPS extraction

Cold 96% ethanol was added to the supernatant obtained from the centrifugation at a 1:3 (v/v) ethanol:supernatant ratio to precipitate the EPS (Breedveld, Zevenhuizen, & Zehnder, 1990). At this stage, it was possible to immediately observe the formation of a precipitate. The mixture was refrigerated at 4 °C for 24 h. After the refrigeration period, the samples were centrifuged once again (10,000 × g, 4 °C, 30 min) to separate the precipitate from the solvent. The precipitate was washed several times with ethanol, and the ethanol was evaporated. The solvent precipitation also achieved a partial purification of the polymer by eliminating the soluble components of the culture media (Castellane & Lemos, 2007; Aranda-Selverio et al., 2010).

The precipitated product was dried using a Hetovac VR-1 lyophilizer until a constant weight was observed, and a precision balance used to verify the quantity of EPS obtained (grams

of EPS per liter of culture medium); the results are presented as the means $\pm$ standard error. The samples were then prepared for reverse-phase high-performance liquid chromatography (RP-HPLC) and rheology analyses.

2.5. Determination of EPS monosaccharide compositions using RP-HPLC

To assess the monosaccharide composition of the EPS produced by the bacterial strains, each raw EPS preparation was analyzed by RP-HPLC using the 1-phenyl-3-methyl-5-pyrazolone monomer chemical identification methodology described by [Fu and O'Neill \(1995\)](#) with modifications.

The EPS samples (1.0 mg) were hydrolyzed with 2 mol L⁻¹ trifluoroacetic acid (200 μ L) in a sealed glass tube (13 $\times$ 100 mm) with screw cap which filled with pure nitrogen gas at 121 °C for 2 h. The hydrolyzed solution was evaporated to dryness under 45 °C and then 2-propanol (500 μ L) was added for further evaporation and complete removal of trifluoroacetic acid. The hydrolysate was used for derivatization.

After hydrolysis, the EPS and monosaccharide standards were pre-derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP), a chemical marker. The reactions were conducted by adding 40 μ L of PMP solution (0.5 mol L⁻¹ in methanol) and 40 μ L of sodium hydroxide solution (0.3 mol L⁻¹) to each tube. The tubes were agitated and incubated at 120 °C for 2 h. The mixture was then neutralized by adding 40 μ L of hydrochloric acid solution (0.3 mol L⁻¹) at to room temperature. For the extraction of monosaccharide derivatives, 0.5 mL of ethyl *tert*-butyl ether was added, and the tubes were agitated for 5 seconds. The layers were separated by centrifugation (5000 $\times$ g, 4 °C, 5 min), and the upper phase (organic layer) was then removed and discarded. This extraction process was repeated five times. The residue was dissolved in water (1.0 mL). The mixture was filtered through 0.45 mm Millipore filter (Waters-Millipore Bedford, MA, USA).

The PMP-labeled monosaccharides were analyzed using the conditions described by [Castellane and Lemos \(2007\)](#) and an HPLC system equipped with a UV-vis spectrophotometer (Shimadzu, model SPD-M10A). The detection wavelength was 245 nm. The monosaccharides glucose, mannose, rhamnose, galactose, glucuronic acid, and galacturonic acid were used as the standards at the following concentrations: 12.50, 25, 50, and 100 μ g mL⁻¹. The retention times (RT) of the monosaccharide compositions of the EPS were determined through a comparison with the chromatograms of each respective standard. The volume of each sample injected into the chromatograph was 20 μ L. Each analysis was performed in duplicate.

2.6. Rheological properties in aqueous medium

Prior to the rheological characterization, the EPS samples were triturated using an Innox triturator until a pulverized solid was obtained. Samples were resuspended in purified water at a temperature of 20 °C, at concentrations of 5 and 10 g L⁻¹. The samples were then stored for at least 24 h to ensure their full hydration. Despite the availability of these polysaccharides, it is necessary to identify and characterize new polysaccharides with specific rheological properties and potential applications. According to [Saude and Junter \(2002\)](#), additional information on the chemical structures and physicochemical characteristics of polysaccharides is required for their use in industry.

The rheology of the polymers in aqueous solution was studied using a controlled stress rheometer (Rheometrics Scientific). The rheological tests were conducted at 25 °C in duplicate. Flow curves were obtained through a program of up-down-up steps, and different shear stress ranges were used for each sample. The ranges

were determined using a shear rate control experiment in which the maximum shear rate value was 100 s⁻¹.

The consistency index 'K' and the flow behavior index 'n' were determined from the power law model ([Steffe, 1996](#)) given by the equation $\eta = K\dot{\gamma}^{n-1}$, where η is the apparent viscosity (Pa s) and $\dot{\gamma}$ is the shear rate (1/s). The value of 'n' was obtained from the slope of the log-log plot of viscosity versus shear rate. The value of 'K' was calculated from the intercept of the same graph.

2.7. Data analysis

All of the determinations reported in this manuscript were performed in triplicate, and the results are presented as the mean values. Results were analyzed by analysis of variance (ANOVA), and means were compared by Tukey's test.

3. Results and discussion

3.1. Phenotypic comparisons between the wild-type and mutant strains of *R. tropici*

In this work, we focused primarily on the choice of media and growth conditions that promoted high *R. tropici* EPS yield because some biological polymers have industrial value in large quantities. We found that *R. tropici* and rhizobial isolates grow and produce measurable EPS using sucrose as a carbon source tested in liquid medium (PSYL). The wild-type strain of *R. tropici* SEMIA 4080, mutant strains (MUTZC3 and MUTPA7) and two rhizobial isolates (JAB1 and JAB6) were cultivated using commercial sucrose as the sole carbon source. This carbon source is inexpensive and easy to obtain and has shown satisfactory results in the production of exopolysaccharides by wild-type strains of *R. tropici* ([Castellane & Lemos, 2007](#)).

Bacteria belonging to the *Rhizobium* genus produce non-negligible amounts of surface polysaccharides. Our observations of *R. tropici* growth and EPS production utilizing sucrose as the sole carbon source showed that the colonies of all of the strains were large, circular, translucent, and mucoid on PSYA (results not shown). The mucoid colonies formed long, viscous filaments when picked with a platinum loop. The colonies were then grown on PSYA containing the fluorescent brightener 28 (calcofluor) and observed through UV illumination at 430 nm. We observed mucoid phenotypic changes in the MUTZC3 strains due to an increased production of polysaccharides; the fluorescent pigment is specific to polysaccharides with β -1 $\rightarrow$ 4 and β -1 $\rightarrow$ 3 linkages ([Wood, 1980](#)), and colonies of the mutant strain showed brighter fluorescence under ultraviolet light. The MUTZC3 mutant strains of *R. tropici* have stronger calcofluor fluorescence than rhizobial isolate JAB1. This indicates that MUTZC3 and JAB6 strains produce more calcofluor-fluorescent exopolysaccharide than rhizobial isolate JAB1 on PSYA medium containing calcofluor (data not shown). No difference in the mucoid phenotype was observed between the wild-type, and the mutant strain (MUTPA7) of *R. tropici*. These results suggest that all strains produce EPS, and the strains of *Phaseolus vulgaris* L. exhibit variation in production of EPS.

3.2. Evaluation of exopolysaccharide production

The dry biomass and isolated EPS were weighed, and the values obtained are presented in [Table 1](#). The MUTPA7 mutant strain of *R. tropici*, rhizobial isolate JAB1 and wild-type (SEMIA 4080) produced 3.94 ± 0.41 g L⁻¹, 3.75 ± 0.30 g L⁻¹ and 2.52 ± 0.45 g L⁻¹ EPS, respectively, whereas the MUTZC3 mutant and rhizobial isolate JAB6 exhibited the best EPS productions (5.52 ± 0.36 and 5.06 ± 0.20 g L⁻¹ EPS, respectively) under the cultivation conditions described in this study ([Table 1](#)). This yield is among the highest

Table 1

Evaluation of the differences in the exopolysaccharide production and cell dry weight between wild-type (SEMIA4080), mutant (MUTZC3 and MUTPA7) strains of *R. tropici* and rhizobial isolates (JAB1 and JAB6).

Strain	EPS	Cell dry weight (CDW) (g L ⁻¹) (mean ± SD)	EPS/CDW
SEMIA 4080	2.52 ± 0.45 ^c	1.14 ± 0.05 ^a	2.21 ± 0.15 ^d
MUTZC3	5.06 ± 0.20 ^a	0.75 ± 0.09 ^c	6.75 ± 0.09 ^a
MUTPA7	3.94 ± 0.41 ^b	0.71 ± 0.12 ^c	5.55 ± 0.25 ^b
JAB1	3.75 ± 0.30 ^b	0.95 ± 0.05 ^b	3.95 ± 0.11 ^c
JAB6	5.52 ± 0.36 ^a	0.78 ± 0.04 ^c	7.08 ± 0.06 ^a

Mean values (±standard deviation) within the same column not sharing a common superscript differ significantly ($P < 0.05$).

yields of EPS reported in *Rhizobium* species, e.g., *R. tropici* CIAT899 exhibited a maximum EPS yield of 4.08 g L⁻¹ under optimized conditions of LMM defined minimal medium with 2% sucrose (Staudt, Wolfe, & Shrout, 2012). Another research team examined two type strains of *R. tropici*, namely BR 322 and BR 520, which were isolated from three nodules of the guandu bean (*Cajanus cajan* cv. Caqui). Both strains were recommended for the production of soil inocula for beans typically grown in Brazil. Furthermore, although the strains were genetically very closely related, they exhibited distinct growth productivities and capacities with EPS productions of 1.13 and 1.89 g L⁻¹, respectively, when cultivated in YML medium (Fernandes, Rohr, Oliveira, Xavier, & Rumjanek, 2009).

However, other known rhizobia, such as *S. meliloti*, are also capable of producing high levels of EPS, e.g., *S. meliloti* SU-47 exhibited a maximum EPS yield of 7.8 g L⁻¹ under optimized conditions of yeast mannitol medium (Breedveld et al., 1990), while the *S. meliloti* strain F exhibited a maximum EPS yield of 2.9 g L⁻¹ under optimized conditions of defined minimal medium with mannitol (Dudman, 1964). Many researchers report that *S. meliloti* strains can be characterized as efficient strains, better in both qualitative and quantitative EPS (Mazur, Król, Marczak, & Skorupska, 2003; Bomfeti et al., 2011; Staudt et al., 2012). As a result, from the present study it is evident that mutant of the *R. tropici* strain MUTZC3 and one rhizobial isolate (JAB6) can be considered as potential microbial cell factories for EPS production. The amount of EPS produced by the two strains (MUTZC3 and JAB6) was not significantly ($P > 0.05$) different.

The cellular biomass production values of the JAB1 (0.95 ± 0.05), JAB6 (0.78 ± 0.04), MUTZC3 (0.75 ± 0.09 g L⁻¹) and MUTPA7 (0.71 ± 0.12 g L⁻¹) strains were lower than that of the wild-type strain (1.14 ± 0.05 g L⁻¹). In general, polymer production is inversely proportional to the bacterial growth index, which suggests a regulatory relationship between the bacterial metabolism and catabolism in which, up to some point on the growth curve, the cells do not invest in carbon skeletons for growth, to the detriment of their metabolic activity (Fernandes Júnior et al., 2010).

Exopolysaccharide production can vary as a function of the growth phase in some bacterial species (Kumari, Ram, & Mallaiah, 2009). Some exopolysaccharides are produced throughout bacterial growth, whereas others are only produced in the late logarithmic or stationary phases (Sutherland, 2001). However, although EPS yields vary with the bacterial growth phase, most studies have shown that the exopolysaccharide composition remains constant throughout the batch cycle of growth (De Vuyst, Vanderveken, Van de Ven, & Degeest, 1998). However, the local environmental chemistry changes during bacterial growth as metabolites and intermediates are consumed and created. Under our testing conditions, *R. tropici* synthesized EPS from the early growth phases through the stationary phase.

We evaluated the relative efficiency of EPS production, which is given by the ratio of the total EPS to the cellular biomass. The rhizobial isolate JAB6 (7.08) and MUTZC3 mutant (6.75) exhibited

Table 2

Comparative monosaccharide composition of EPS (%) produced by the wild-type (SEMIA4080), mutant (MUTZC3 and MUTPA7) strains of *R. tropici* and rhizobial isolates (JAB1 and JAB6)^a.

Type of exopolysaccharide	Composition (%)					
	Man	Rha	GlcA	GalA	Glc	Gal
EPS of SEMIA 4080 (EPSWT)	0.86	2.58	8.6	tr	55.48	32.47
EPS of MUTZC3 (EPSC3)	0.74	2.60	tr	2.60	53.53	40.52
EPS of MUTPA7 (EPSPA7)	1.15	2.31	tr	2.70	54.63	39.19
EPS of JAB1 (EPSJ1)	1.49	2.49	5.97	tr	60.70	29.35
EPS of JAB6 (EPSJ6)	2.68	0.60	3.57	tr	54.17	38.99

^a Man, mannose; Rha, rhamnose; GlcA, glucuronic acid; GalA, galacturonic acid; Glc, glucose; Gal, galactose; tr = trace.

the highest EPS production efficiency, followed by the MUTPA7 mutant (5.55), rhizobial isolate JAB1 (3.95) and wild-type strain (2.21). It is common to find variable EPS productions between bacteria, even among bacteria of the same genus cultivated under the same conditions, as shown for *Rhizobium* (Kumari et al., 2009), *Xanthomonas* (Antunes, Moreira, Vendruscolo, & Vendruscolo, 2003; Rottava et al., 2009), and *Sphingomonas* (Berwanger et al., 2007).

3.3. EPS monomer characterization

After hydrolysis and PMP derivatization, the EPS were characterized, and the monomer contents were quantified by HPLC; the results are summarized in Table 2, and the HPLC chromatograms are presented in Fig. 1. The analysis of the EPS monosaccharide composition shows that glucose and galactose are the most abundant monomers, and small amounts of mannose, rhamnose, glucuronic acid, and galacturonic acid are also present (Table 2). Many EPS components are water-soluble biopolymers composed of a wide range of monomers and may contain as many as nine different sugar residues in repeating units (Castellane & Lemos, 2007; Monteiro et al., 2012; Sutherland, 2001).

Interestingly, the EPS produced by the MUTZC3 and MUTPA7 mutant strains of *R. tropici*, which are identified designated as EPSC3 and EPSPA7, respectively, included small quantities of mannose (0.74 and 1.15%), rhamnose (2.60 and 2.31%), and galacturonic acid (2.60 and 2.70%) and trace amounts of glucuronic acid. In contrast, the EPS produced by the wild-type strain SEMIA 4080, rhizobial isolates JAB1 and JAB6, which are identified designated as EPSWT, EPSJ1 and EPSJ6, respectively, included small quantities of mannose (0.86%, 1.49% and 2.68%), rhamnose (2.58%, 2.49% and 0.60%), and glucuronic acid (8.6%, 5.97% and 3.57%) and trace amounts of galacturonic acid (Table 2). These data are similar to the findings reported by Castellane and Lemos (2007), who found that an EPS obtained from the cultivation of *R. tropici* SEMIA 4077 was primarily composed of glucose and galactose with trace amounts of mannose and rhamnose.

Kaci, Heyraud, Barakat, and Heulin (2005) isolated and characterized an EPS produced by a type strain of *Rhizobium* from arid earth as a polymer of glucose, galactose, and mannuronic acid in the molar proportion of 2:1:1. In general, EPS synthesized by fast-growing rhizobia (e.g., *S. meliloti* and *R. leguminosarum*) are composed of octasaccharide repeating units, in which glucose is a dominant sugar component. In *R. leguminosarum* bv. *trifolii*, an EPS subunit is composed of seven sugars, none of which is galactose (Amemura, Harada, Abe, & Higashi, 1983). In *R. leguminosarum* bv. *viciae* 248, the EPS subunit has an additional glucuronic acid (Canter-Cremers et al., 1991). Low and high molecular weight fractions of EPS were reported to be produced by *S. meliloti* and *R. leguminosarum* (Mazur et al., 2003).

As shown in Table 2, the EPS produced by the wild-type strain SEMIA 4080, JAB1 and JAB6 isolates contain small quantities of

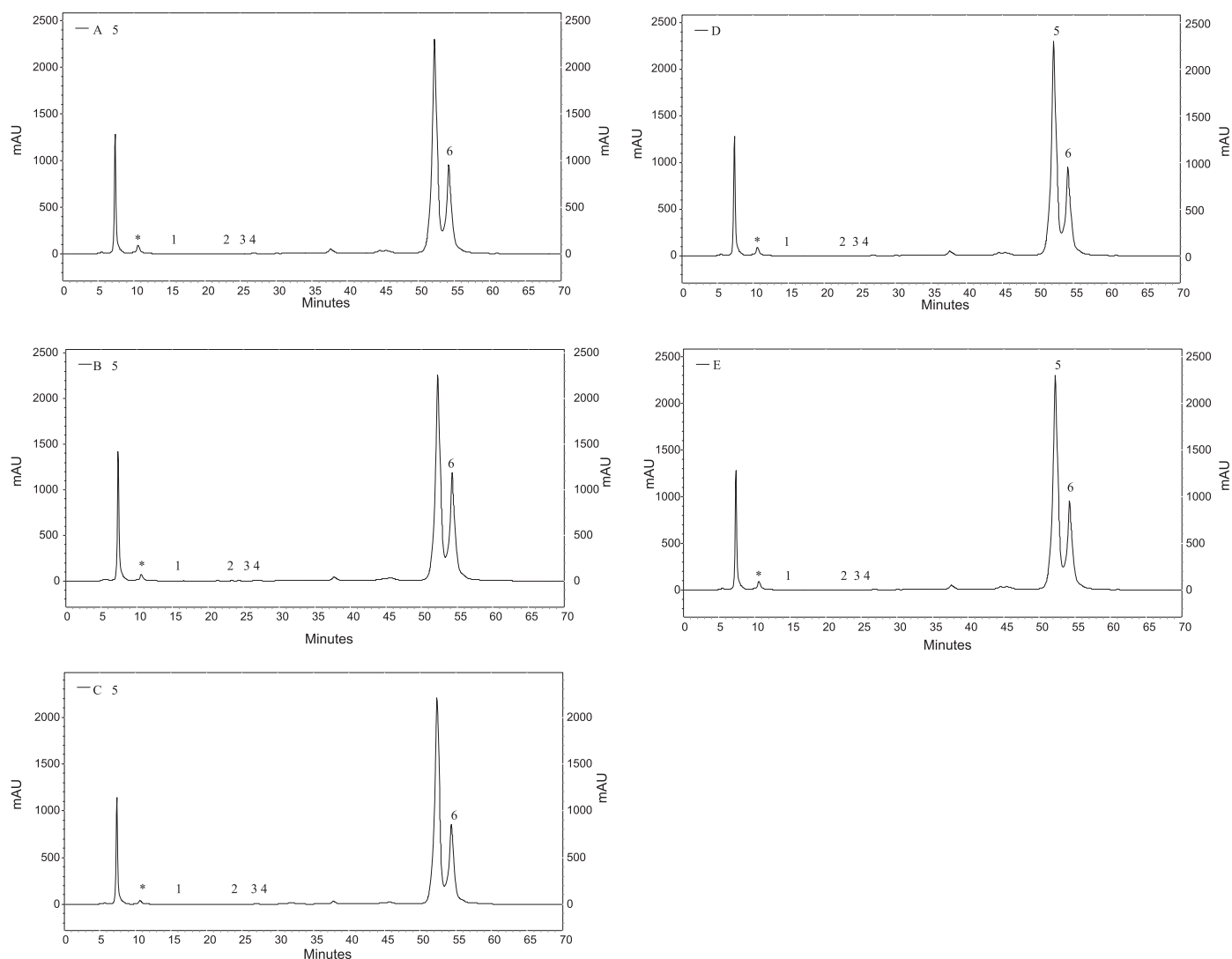


Fig. 1. Monosaccharide analysis of the EPS samples by HPLC of the PMP derivatives of the acid hydrolysate of the EPS: (A) *Rhizobium tropici* SEMIA 4080, (B) the MUTZC3 mutant strain, (C) the MUTPA7 mutant strain, (D) rhizobial isolate JAB1 and (E) rhizobial isolate JAB6. The chromatographs of the EPS from show peaks for (*) 1-phenyl-3-methyl-5-pyrazolone residue, (1) mannose, (2) rhamnose, (3) glucuronic acid, (4) galacturonic acid, (5) glucose, and (6) galactose.

glucuronic acid (8.6%, 5.97% and 3.57%, respectively), and EPSC3 and EPSPA7 showed traces of glucuronic acid. [Staehelin et al. \(2006\)](#) also found a very small quantity of glucuronic acid in the EPS of the *Rhizobium* sp. type strain NGR234. The presence of acid monosaccharides (glucuronic and galacturonic acid), even in low concentrations, renders an EPS acidic, and its accumulation makes the heteropolysaccharide highly anionic. Therefore, such EPS can act as ion-exchange resins and thus concentrate minerals and nutrients near the cell ([Whitfield, 1988](#)). The presence of glucuronic and pyruvic acid increases the ionization of the material, thereby promoting alterations in its molecular conformation and increasing its solubility ([Diaz, Vendruscolo, & Vendruscolo, 2004](#)).

3.4. Rheological properties in aqueous medium

The flow curves of the exopolysaccharide solutions obtained from the wild-type, mutant strains of *R. tropici* and rhizobial isolates are shown in [Fig. 2](#). As shown in [Fig. 2](#), solutions of the pure exopolysaccharides EPSWT, EPSC3, EPSPA7, EPSJ1 and EPSJ6 showed non-Newtonian behavior at shear rates between 0.1 and 100 s⁻¹. Previous studies evaluating the EPS from rhizobial isolates have shown that this type of polymer generally

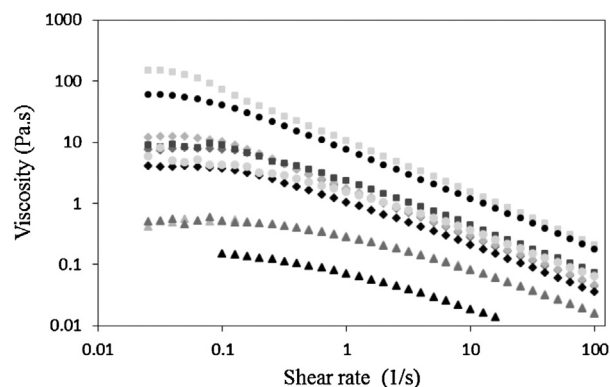


Fig. 2. Flow curves of solutions of the exopolysaccharides from the wild-type strain of *Rhizobium tropici* (SEMIA4080) and from the mutant (MUTZC3 and MUTPA7) strains at two different concentrations. These flow curves were measured at 25 °C. The symbols represent the following: ♦, ♦, □, and ● for EPSWT, EPSC3, EPSPA7, EPSJ1, and EPSJ6, respectively, at 10 g L⁻¹; ▲, ▲, ▲, and ● for EPSWT, EPSC3, EPSPA7, EPSJ1, and EPSJ6, respectively, at 5 g L⁻¹.

Table 3

Coefficients of the power law model for EPS solutions at two different concentrations.

Type of exopolysaccharide	<i>K</i>	<i>n</i>
EPS of SEMIA 4080 (EPSWT) (5 g L ⁻¹)	0.29 ± 0.02 ^e	0.41 ± 0.03 ^a
EPS of SEMIA 4080 (EPSWT) (10 g L ⁻¹)	1.71 ± 0.09 ^c	0.22 ± 0.01 ^b
EPS of MUTZC3 (EPSC3) (5 g L ⁻¹)	0.30 ± 0.01 ^e	0.40 ± 0.04 ^a
EPS of MUTZC3 (EPSC3) (10 g L ⁻¹)	1.77 ± 0.12 ^c	0.22 ± 0.01 ^b
EPS of MUTPA7 (EPSPA7) (5 g L ⁻¹)	0.17 ± 0.01 ^f	0.48 ± 0.08 ^a
EPS of MUTPA7 (EPSPA7) (10 g L ⁻¹)	1.03 ± 0.02 ^d	0.29 ± 0.02 ^b
EPS of JAB1 (EPSJ1) (5 g L ⁻¹)	2.1 ± 0.14 ^c	0.25 ± 0.01 ^b
EPS of JAB1 (EPSJ1) (10 g L ⁻¹)	10.2 ± 0.25 ^a	0.16 ± 0.02 ^c
EPS of JAB6 (EPSJ6) (5 g L ⁻¹)	1.9 ± 0.09 ^c	0.26 ± 0.04 ^b
EPS of JAB6 (EPSJ6) (10 g L ⁻¹)	7.3 ± 0.39 ^b	0.20 ± 0.06 ^b

Mean values (±standard deviation) within the same column not sharing a common superscript differ significantly ($P < 0.05$).

Flow behavior index, *n*, and consistency coefficient, *K*, obtained by the Ostwald-de Waele model: $\eta = K\dot{\gamma}^{n-1}$.

exhibits non-Newtonian behavior and is pseudoplastic (Kaci et al., 2005; Aranda-Selverio et al., 2010). Pseudoplastic or shear thinning behavior has been reported for other biopolymers with industrial applications, such as xanthan (Katzbauer, 1998), gellan gums (Drevet, Monot, Ballerini, Lecourtier, & Choplin, 1994) and succinoglycan (Kido, Nakanishi, Norisuye, Kaneda, & Yanaki, 2001).

The rheological behaviors of materials may be described using models that describe how the surface tension varies with the deformation rate. The mathematical models that are most commonly utilized for food systems are the Ostwald-de Waele (power law), Casson, Herschel–Bulkley, and Mizrahi–Berki models. The first two models use mathematical equations with two parameters, whereas the others use equations with three parameters (Haminiuk, Sierakowski, Izidoro, & Masson, 2006). The Ostwald-de Waele model allowed the best adjustments to the solutions of 5 and 10 g L⁻¹ exopolysaccharides produced by the wild-type *R. tropici* SEMIA 4080, the mutant (MUTZC3 and MUTPA7) strains and rhizobial isolates (JAB1 and JAB6) at a temperature of 25 °C. The power law model is easy to use and is ideal for pseudoplastic, relatively mobile fluids, such as weak gels and low-viscosity dispersions. The coefficients of this model for all of the solutions analyzed are presented in Table 3. The consistency coefficient '*K*' describes the overall range of viscosities across the modeled portion of the flow curve.

The values of both the consistency index (*K*) and flow behavior index (*n*) were significantly dependent ($P < 0.05$) on the strain (Table 3). The '*K*' value indicated a progressive increase in viscosity with an increase in the EPS concentration for all EPS tested. The rheological profile was very close to that of the biopolymers produced by the wild-type *R. tropici* SEMIA 4080 and the MUTZC3 mutant strain at the concentration of 5 g L⁻¹ (Fig. 2 and Table 3). The EPS produced by the rhizobial isolates JAB1 and JAB6 showed higher *K* values than the EPSWT and EPSC3, indicating more shear-resistant nature.

As shown in Table 3, the values of '*K*' obtained for the EPS produced by the MUTZC3 and SEMIA 4080 strains were higher than those found for the MUTPA7 mutant, which indicates that the EPSWT and EPSC3 solutions at concentrations of 5 and 10 g L⁻¹ are much more viscous than the polysaccharide excreted by the MUTPA7 mutant strain. The exponent *n* (known as the power law index) has values between 0 and 1 for a shear thinning fluid, whereas more pseudoplastic products exhibit lower values of *n* (close to zero) (Steffe, 1996).

These results obtained in this study are similar to those obtained by Aranda-Selverio et al. (2010), who reported that EPS solutions produced by rhizobia isolated from *Phaseolus vulgaris*, *Leucaena leucocephala* v. *cunnie*, *Pisum sativum*, and *A. pintoi* exhibit pseudoplastic behavior. The aqueous solutions of the EPS produced by

three different strains of bacteria of the *Rhizobium* genus behaved as non-Newtonian fluids. A decrease in the surface tension accompanied by an increase of the surface index results in a lower apparent viscosity, which means that the solutions are pseudoplastic fluids (Navarro, 1997). The rheological analyses demonstrate that these polysaccharide solutions exhibit pseudoplastic fluid behavior and may thus be utilized as thickening agents with polyelectrolytic properties. However, despite this pseudoplastic behavior, the viscosity of the solutions of the EPS from different rhizobial type strains may vary even though they exhibit the same surface indexes (Aranda-Selverio et al., 2010), suggesting that these polymers may have different biotechnological applications.

4. Conclusions

The exopolysaccharides produced by the different mutants of *R. tropici* showed subtle differences in their monosaccharide compositions (primary structure) compared with the wild-type strain, which may be sufficient to cause alterations in the secondary structures or conformations of the molecules. This hypothesis is supported by the results of the rheological analyses of each of the studied EPS. All of the biopolymers produced by these strains demonstrated pseudoplastic behavior.

Because the use of rhizobia in the commercial production of gum has not been studied, rhizobia may be considered highly promising unexplored sources of microbial polysaccharides for industrial applications. These bacteria exhibit great morphological, physiological, genetic, and phylogenetic diversity and can be a valuable source for the screening of strains with specific properties. Because none of the α - and β -rhizobia discovered to date has been shown to be pathogenic, this group can be generally characterized as an unexplored source of microbial EPS with excellent potential for use in industrial applications and as soil-stabilizing agents. This may represent a potential opportunity for the bio-inoculant producing industries in Brazil as best alternative activity during the non crop season of the soybean and common bean. The MUTZC3 mutant and rhizobial isolate JAB6 exhibited the best EPS productions. While the EPS produced by the rhizobial isolates JAB1 and JAB6 showed higher consistency index values than the EPS produced by the mutant strains MUTZC3, indicating more shear-resistant nature. Therefore, we concluded that these strains could be exploited for the large-scale commercial production of Rhizobial polysaccharides.

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