

## Changes in *Bradyrhizobium* sp. Polysaccharides and in Peanut Root Nodule Polyamines Produced by the Fungicide Mancozeb

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### Abstract

We have previously shown that the fungicide Mancozeb reduced *Bradyrhizobium* sp. growth by 50%, produced biochemical alterations such as changes in membrane composition and polyamine contents, and affected the peanut root-bacteria interaction in greenhouse experiments. In this work we show the variations caused by the fungicide on the molecules related with the peanut-bacteria interaction such as lipopolysaccharides (LPS) and cyclic  $\beta$ -glucans. Furthermore, the changes on peanut root nodule polyamines induced by Mancozeb are analyzed.

**Keywords:** *Bradyrhizobium* sp., Mancozeb, peanut root nodules, lipopolysaccharides, cyclic  $\beta$ -glucans, polyamines

### 1. Introduction

Fungicides are used to protect seeds and seedlings from pathogenic microorganisms. The seed-applied chemicals are often non-specific in their lethal action, and they may limit the symbiotic process affecting survival and viability of rhizobia. Conclusions on compatibility of these bacteria with

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fungicides are controversial. Variations in test methods, neglect of important variables, and insufficient concern for the quantitative aspects of *Rhizobium* survival in presence of fungicides have contributed to these conflicting reports (Hashem et al., 1997).

*Rhizobium*-plant interaction is very specific, and usually the defined species of *Rhizobium* can form nodules on a limited number of hosts. In several *Rhizobium* species the genetic control of nodulation (*nod* genes) and nitrogen fixation (*nif* genes) as well as the mechanisms of recognition are known (Fisher and Long, 1992). The rhizobial genes essential for infection and nodule formation also include genes controlling synthesis of surface polysaccharides: exopolysaccharides (EPS), lipopolysaccharides (LPS) and  $\beta$ -glucans. Bacterial mutants defective in synthesis of these compounds are mostly also defective in symbiotic properties. Surface components of *Rhizobium* can play a role as signal molecules on different steps of the nodulation process as well as in protection of bacteria from host defense mechanisms. Most of mutants defective in surface polysaccharides form ineffective nodules with no or abortive infection threads (Kannenberg and Brewin, 1994; Carlson et al., 1992).

Several roles for lipopolysaccharides and cyclic  $\beta$ -glucans in the symbiosis of legume plants with rhizobia have been proposed. Lipopolysaccharides are on the outer membrane of Gram-negative bacteria. LPS consists of lipid A, core oligosaccharide (core) and O-antigenic polysaccharide (O-chain). LPS is important for the process of infection by some rhizobial species; lipopolysaccharide mutants of *Rhizobium leguminosarum*, *Rhizobium etli* or *Bradyrhizobium japonicum* which lack O-antigen can not induce nitrogen fixing nodules (Noel et al., 1992; Brewin et al., 1993). Cyclic  $\beta$ -glucans are unique molecules that are found almost exclusively in bacteria of the Rhizobiaceae family. The glucans are cyclic homopolymers of 17 to 24  $\beta$ -D-glucopyranose units. Reaching concentrations as high as 5 to 20% of the total cellular dry weight under certain culture conditions, the cyclic  $\beta$ -glucans are major cellular constituents (Breedveld and Miler, 1994). It has been suggested that they are involved in the special interaction between these bacteria and plants (Batley et al., 1987).

Legume root nodules, which fix atmospheric nitrogen to form ammonia, are complex tissues differentiated from the root as a result of legume-*Rhizobium* symbiotic associations. Ozawa and Tsuji (1993) hypothesized that nodule polyamines might have a regulatory role on the proliferation of bacteroid cells within root nodules, since the polyamines (organic polycations found in all the living cells) are known to stimulate plant growth and development (Slocum et al., 1984). Smith (1977) first reported the presence of high concentration of an unusual triamine, homospermidine, in *Rhizobium* and root nodules from lupin, pea and faba bean. However, the localization, function and metabolism of polyamines within the root nodules are not yet fully understood.

The fungicide Mancozeb is extensively used on peanut seeds and in various field crops to control many fungal diseases caused by a wide range of pathogens throughout the world (Worthing and Hance, 1991). Peanut (*Arachis hypogaea* L.) is an important legume grown in Argentina, with 98% of its production concentrated in the province of Córdoba. Argentina is ranked as the second largest peanut exporter in the world. We have previously shown that the fungicide affects the bacteria-peanut root interaction in growth chamber experiments (Castro et al., 1997) and causes alterations on *Bradyrhizobium* sp. polyamine contents as well as an increment of lipopolysaccharide contents in pure culture (Fabra et al., 1998).

In order to analyze the mechanism involved in the harmful effect of Mancozeb observed on the bacteria-peanut root interaction, the present work was planned to study the effect of the fungicide on *Bradyrhizobium* sp. surface polysaccharides that provide important functions during the plant infection process, as well as to determine the action of the fungicide on compounds related with the cellular growth such as polyamines in peanut root nodule.

## 2. Materials and Methods

### *Chemical reagents*

The fungicide Mancozeb (ethylenebis-dithiocarbamate) was supplied by Rohm and Haas Co., Argentina. Other chemicals were obtained from Sigma Chemical, St. Louis, MO or from Merck Química, Argentina. Homospermidine was gently provided by Dr. S. Fujihara (National Agriculture Research Center, Tsukuba, Japan).

### *Bacterial strain and growth conditions*

The strain used in this study was *Bradyrhizobium* sp. USDA 3187 obtained from IPAGRO, Brazil. It is a recommended microsymbiont for peanut inoculation. Stock cultures were maintained on YEMA (Vincent, 1970) slants and transferred once a month. Purity was assured by routine plating on YEMA supplemented with Congo red and observing uniform colonies. Primary cultures in 10 ml quantities (YEM) were started from agar slants and incubated at 28°C, with shaking until the early logarithmic phase. These cultures provided inocula for experimental cultures which were also grown with shaking at 28°C.

Bacteria were treated with 2 mg/ml Mancozeb in the culture medium from the beginning of incubation. This dose is equivalent to that recommended for field conditions (200 g/100 kg seeds).

### *Lipopolysaccharide determination*

LPS were extracted from cellular pellets obtained from bacteria grown in YEM medium for 8 h by the hot-phenol-water method (Westphal and Jann, 1965). Samples of LPS were applied to a 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) as described previously by Laemmli (1970). This procedure was repeated three times. After electrophoresis, gels were silver-stained (Tsai and Frash, 1982).

### *Glucan determination*

To isolate glucans, cell pellets were extracted with 75% ethanol at 70°C for 30 min (Breedveld et al., 1995). After centrifugation at 10,000 × g for 10 min, the alcoholic supernatant was concentrated under vacuum and the residue was dissolved in water. Quantitative determination was carried out by the anthrone method (Dische, 1962). Aliquots containing 5 µg of glucans were spotted onto TLC plates (Silica gel G 60, Sigma) and chromatographed with ethanol-butanol-water (5:5:4). For visualization of the different bands, the TLC plates were sprayed with 5% H<sub>2</sub>SO<sub>4</sub> in methanol and heated at 120°C for 10 min.

### *Polyamine determination*

Root nodules were collected from peanut plants at the flowering growth stage derived from Mancozeb-treated or untreated peanut seeds (*Arachis hypogaea* cv. Florunner) under field conditions. The field experiment was conducted on the Universidad Nacional de Río Cuarto plot, in a sandy loam Typic Hapludoll soil (pH 6.2; organic matter 1.8%).

The nodules were washed under running tap water and conserved with a desiccant (CaCl<sub>2</sub>) at -30°C until the analysis of polyamines. Frozen nodules were gently crushed with a pestle and mortar in a saline solution (0.05 M K<sub>2</sub>HPO<sub>4</sub>, 0.05 M KH<sub>2</sub>PO<sub>4</sub>, 0.002 M MgSO<sub>4</sub>, 0.14 M NaCl, pH 7.2) and then centrifuged at 10,000 × g for 10 min. The resulting pellet was used for polyamine determination employing the technique of Smith and Best (1977). The pellet was treated with 5% trichloroacetic acid and centrifuged at 420 × g. The resulting supernatant was neutralized with sodium bicarbonate and kept in darkness for 16 h after the addition of 0.2 ml dansyl chloride solution (30 mg/ml); 0.1 ml of a proline solution (150 mg/ml) was added to each sample, which was then maintained at room temperature for 30 min and subsequently dried with a current of warm air. The dry residue was dissolved in 0.1 ml of water, 0.2 ml of toluene was added and, after mixing for a few minutes, the



sample was centrifuged. The toluene layer was separated and a fraction of it placed on a TLC plate (Silica gel G 60, Sigma). The chromatography was run for 2 h using a chloroform/triethylamine solvent (5:1). The polyamines were detected by means of UV light. Spots were extracted with acetone and the concentration determined with a spectrofluorometer (excitation 359 nm and emission 500 nm). The results were compared with dansylated polyamine standards.

#### *Protein determination*

Proteins were determined by the method of Bradford (1976) with bovine albumin as standard.

#### *Statistical analysis*

The data were analyzed statistically by the Student t-test, and a level of  $p < 0.05$  was accepted as significant.

### **3. Results and Discussion**

#### *Effect of Mancozeb on molecules related to the plant-bacteria interaction*

It has been shown that some fungicides may have a bacteriocidal rather than bacteriostatic action and directly affect survival of rhizobia in soil, reducing the level of infection and hence nodulation (Lal, 1988). We demonstrated that Mancozeb decreased *Bradyrhizobium* sp. growth rate by 50% and produced a negative effect on the bacteria-peanut interaction in greenhouse experiments (Castro et al., 1997). Several recent studies indicate that rhizobial lipopolysaccharides are involved in the nodule development, especially in hosts that form determinate nodules, such as peanut (Hirsch, 1992). The isolation of mutant bacteria of the genera *Rhizobium* and *Bradyrhizobium* with altered LPS proved the direct relationship between the structure of these molecules and its symbiotic properties (Dazzo et al., 1991; Glowacka et al., 1996). Thus, for a successful invasion, rhizobial *lps* gene is required to contribute to host-specific nodulation (Noel, 1992; Gray et al., 1992).

Fabra et al. (1998) demonstrated that *Bradyrhizobium* sp. LPS content increased by 45% as a consequence of Mancozeb stress. It would indicate changes in the membrane permeability with a resulting increase in the cellular hydrophobicity. In addition, LPS-banding patterns indicated alterations in the core region of LPS (Fig. 1). Bhat and Carlson (1992) reported that changes in LPS structure or production are related to adaptation to different environmental

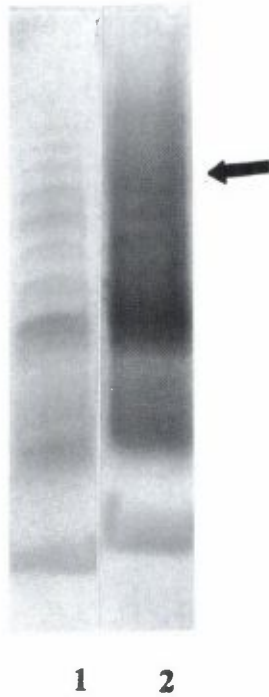


Figure 1. Electrophoretic analysis of the LPS of *Bradyrhizobium* sp. visualized with silver stain. Lane 1: Control; lane 2: Mancozeb. Arrow indicates the main change observed.

conditions such as pH, oxygen concentration and osmotic pressure. The modifications of *Bradyrhizobium* sp. LPS would be a response to chemical stress. Furthermore, one can also assume that these changes may be contributing to the alteration in the bacteria-peanut interaction observed.

Cyclic  $\beta$ -glucans have been shown to provide important functions both for the free-living forms of these bacteria and during the process of plant infection. The *ndv* mutants of *Rhizobium* species have provided the most insight concerning the possible roles for the cyclic  $\beta$ -glucans in the Rhizobiaceae. Mutants with alterations at these loci induced the formation of nodule-like structures that were lacking infecting bacteria or a visible infection thread (Dylan et al., 1986). Other authors reported that nodules induced by *Bradyrhizobium japonicum ndvB* mutants showed normal tissue differentiation but they were defective in nitrogen fixation ability (Dunlap et al., 1996). Data obtained from pseudorevertants revealed that cyclic  $\beta$ -glucans are not essential for nodulation, but clearly play an important role (Dylan et al., 1990). It should be considered that cyclic  $\beta$ -glucans may function during several stages of plant

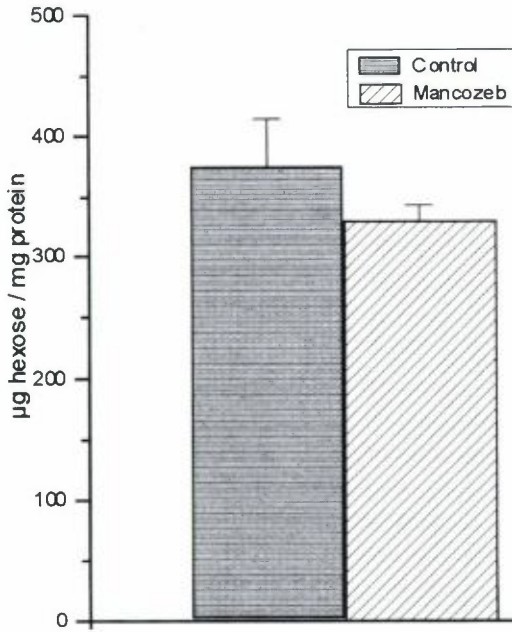


Figure 2. Effect of Mancozeb on cyclic  $\beta$ -glucan contents in *Bradyrhizobium* sp. Data are means  $\pm$ S.E. of three determinations

infection. These include roles in conferring host specificity, interactions with plant metabolites, roles during attachment to plant cell and suppression of host defense responses (Breedveld and Miller, 1994).

There was no significant difference in the content of cyclic  $\beta$ -glucans found in *Bradyrhizobium* sp. in the presence or absence of Mancozeb (Fig. 2). However, the analysis of the cyclic  $\beta$ -glucan pattern demonstrated changes such as the appearance of a Rf 2.3 band and the disappearance of a Rf 1.6 band (Fig. 3). This result may indicate that the integrity of the outer membrane of *Bradyrhizobium* sp. was modified and perhaps could also be the cause of membrane permeability alterations. In turn, these alterations could be responsible for the failure observed in the peanut-bacteria interaction.

#### *Effect of Mancozeb on molecules related to cellular growth*

It has generally been accepted that in plants (Flores and Galston, 1982) and in microorganisms (Fujihara and Yoneyama, 1994), various abiotic stresses bring about a rapid change in the cellular polyamine level, especially that of

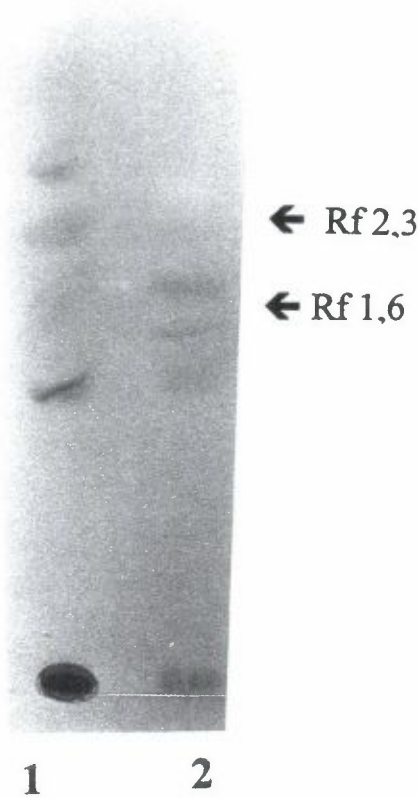


Figure 3. TLC analysis of glucans isolated from *Bradyrhizobium* sp. grown in control medium (lane 1); in treated medium (lane 2). Arrows indicate the main alterations observed in the pattern of the treated group.

putrescine. Increases in the polyamine content was observed in rhizobia undergoing abiotic stresses such as saline or acidic stress (Fujihara and Yoneyama, 1993, 1994). In relation to polyamines found in legume root nodules and based on the ratio of homospermidine to spermidine, Fujihara et al. (1994) demonstrated that each legume had its own polyamine metabolism in root nodules. On the other hand, profiles of nodule polyamine composition were basically similar within the same species as long as root nodules were collected from plants at a physiologically similar growth stage. In this way, adzuki bean root nodules were characterized by their especially high cadaverine content and high homospermidine/spermidine ratio.

The effect of Mancozeb on peanut root nodule polyamine contents is shown in Table 1 and Fig. 4. The putrescine and spermidine content of the nodules obtained from Mancozeb-treated seeds was increased about 4-fold and the



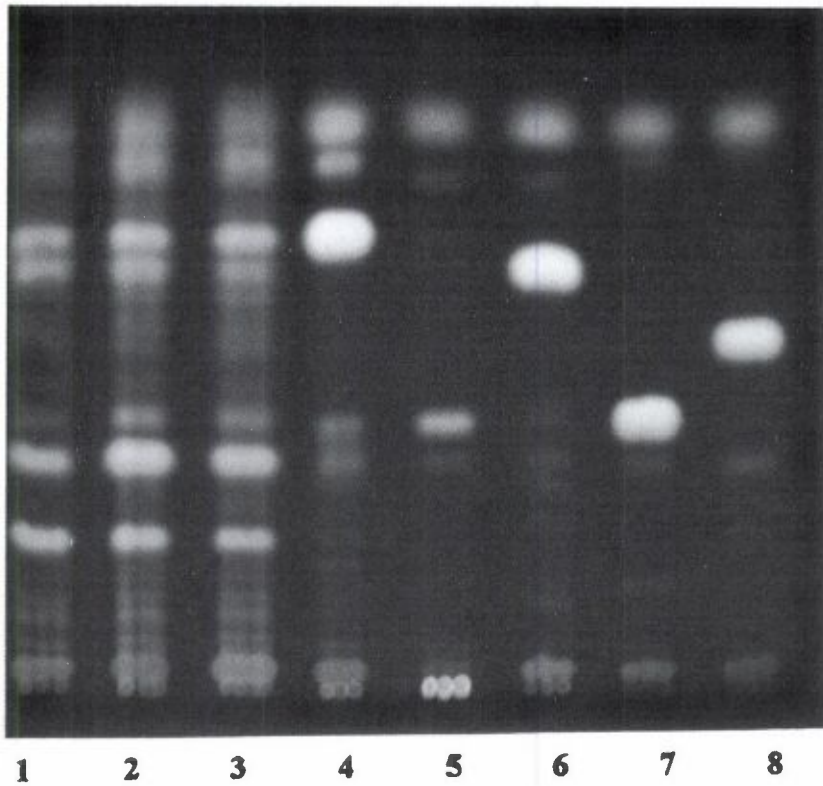


Figure 4. TLC plate of polyamine contents in peanut root nodule viewed and photographed under UV light. Lanes 1-2: Mancozeb treated samples; lane 3: control samples; lane 4: homospermidine standard; lane 5: agmatine standard; lane 6: spermidine standard; lane 7: putrescine standard; lane 8: cadaverine standard.

Table 1. Effect of Mancozeb on polyamine contents in peanut root nodules

Treatments	Polyamine contents ( $\mu\text{g}/\text{mg}$ protein)			
	Putrescine	Spermidine	Homospermidine	Cadaverine
Control	$1.90 \pm 0.48$	$1.69 \pm 0.26$	$1.15 \pm 0.05$	nd
Mancozeb	$3.77 \pm 0.30^*$	$10.66 \pm 2.16^{**}$	$2.12 \pm 0.54$	nd

Data are means  $\pm$  S.E. of two determinations. \* $p < 0.05$ ; \*\* $p < 0.025$ ; nd = not detected.

polyamine cadaverine was not detected either in control nodules or in treated ones. Furthermore, a low homospermidine content was detected in both of them. This triamine is thought to be mainly derived from bacteroid tissue within the nodules (Smith, 1977; Hamana et al., 1990) whereas root cells of host legumes usually produce spermidine but not homospermidine (Fujihara et al., 1986). Taking into account that homospermidine is derived from the bacteroids, it is possible to assume that changes observed in nodule polyamine content are related to an effect of the fungicide on the metabolism of these amines in legume tissue.

In conclusion, on the basis of the results obtained in this study, it is possible to establish a relationship between the changes produced by Mancozeb on *Bradyrhizobium* sp., LPS and cyclic glucans. In an earlier work a harmful effect of this fungicide on the peanut-bacteria interaction was demonstrated.

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