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Short communication

Role of rhizobial EPS in the evasion of peanut defense response during the crack-entry infection process

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Abstract

Roles of rhizobial exopolysaccharides (EPS) in symbiotic nodulation have been most thoroughly studied in legumes infected by the infection thread (IT) mechanism. Peanut (*Arachis hypogaea* L.) differs from other legumes in that rhizobial penetration and spreading inside the nodule occur without IT formation but rather by crack-entry infection. By using a defined mutant (NET30-M1024) affected in the EPS production, we have previously shown that peanut symbionts require these molecules for efficient nodulation. In this work, we monitored the relationship between the symbiotic behavior of this mutant and the EPS level production, and evaluated *ex planta* if these molecules could play a role in protecting the microsymbiont against plant defense reactions.

Keywords: Crack entry; Exopolysaccharides; Plant defense system

Legumes are vital components of agricultural systems because of their ability to associate symbiotically with rhizobia that fix nitrogen from the atmosphere and contribute to plant nutrition.

Efficient nodulation is controlled, among other factors, by the rhizobial cell surface composition. Several studies in the literature indicate that exopolysaccharides (EPS) are required for the formation of N-fixing nodules in legumes infected by the infection thread (IT) mechanism (Gage, 2004). Putative roles that were proposed for rhizobial EPS include specific recognition and active signaling during IT colonization and elongation (Laus et al., 2004, 2005; Battisti et al., 1992; Gonzáles et al., 1996a, b; Cheng and Walker, 1998), organization of the root hair cytoskeleton and redirection of tip growth of root hairs (Rolfe et al., 1996; van Workum et al., 1998; Pellock et al., 2000) and suppression of the host defense response (Niehaus et al., 1993; Parniske et al., 1994; Skorupska et al., 1995; Rolfe et al., 1996; Wielbo et al., 2004). Plants have evolved several defense mechanisms against bacterial infection, such as antimicrobial compounds, phytoalexins and reactive oxygen species (Skorupska et al., 2006). The oxidative burst is an early response in plant defense reactions toward pathogens. A massive production of reactive oxygen species (ROS), namely superoxide (O_2^-) and hydrogen peroxide (H_2O_2) is produced as a plant defense response against avirulent parthogens (Santos et al., 2001). Hydrogen peroxide can have a direct antibiotic activity against invading microorganisms, increase cross-linking of proteins in the plant cell wall and also induce the expression of genes related to plant defense (Mauch-Mani and Métraux, 1998; Shaw and Long, 2003). It was reported that alfalfa responds to infection with *Sinorhizobium meliloti* by production of O_2^- and H_2O_2, suggesting that, at the early stages of infections, the oxidative burst appears to be involved in the control of infection and nodulation (Santos et al., 2001). Furthermore, it was observed that reactive oxygen production by *Medicago truncatula* decreases in the presence of Nod factors, a symbiotic signal produced by rhizobia (Shaw and Long, 2003). Thus, it appears that plant pathogenesis and symbiosis are variations on a common theme. Symbiotic bacteria are initially recognized as intruders but then prevent or overcome plant defense

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reactions inhibiting signaling pathways leading to the deleterious defense cascade (Hérouart et al., 2002).

Root colonization in peanut (*A. hypogaea* L.) involves the crack entry and intercellular spreading, without the IT formation (Boogerd and van Rossum, 1997). In a previous work, we characterized a Tn5-generated peanut symbiont mutant (designated NET30-M1024) that produced less EPS than the parental strain (NET30-wt). The amounts of EPS produced by this mutant varied with the carbon source in the culture media. Severe alterations in nodule development were reported when peanut roots were inoculated with the EPS mutant producing 46% of wild-type EPS production. Only a few bacteroids and abundant empty cells containing large vacuoles were observed in the nodules developed. These results suggested that EPS is a critical factor in the symbiotic interactions between rhizobia and legumes infected by crack entry (Morgante et al., 2005).

In the present study, the NET30-M1024 mutant symbiotic phenotype was further characterized when presenting a higher EPS yield (i.e. 57% of the wild type). We also investigated if the impaired symbiotic phenotype was related with the role of EPS in the protection against the host defense reactions as proposed by Leigh and Coplin (1992).

A. hypogaea L. cv Tegua seeds were disinfected as described by Somasegaran and Hoben (1994). After germination, seeds were placed in pots containing sterile vermiculite (one seedling per pot) and grown in a greenhouse as described by Taurian et al. (2002). Three milliliters of early stationary NET30-wt or NET30-M1024 cultures in MM1 medium (fructose $1\,g\,l^{-1}$; $80\,\mu g\,ml^{-1}$ spectinomycin when required) were centrifuged at 10,000 rev min^{-1} for 10 min and resuspended in 3 ml of fresh medium to give a concentration of 10^8 ufc ml^{-1}. This suspension was added to each pot containing a 7 days peanut seedling. Control uninoculated plants were also included. Eight pots were used for each treatment. Sixty days after inoculation, plants were harvested to determine percentage of nodulated plants, number and dry weight of nodules. Nitrogen fixation was indirectly evaluated on the basis of plant dry weight production and nitrogen content of plant shoot.

In order to investigate if EPS are involved in the suppression of host defense reactions, the H_2O_2 sensitivity of NET30-M1024 was assayed by a simple and sensitive method described by D'Haeze et al. (2004). Strain NET30-wt or NET30-M1024 was replica plated as single colonies on Harada glucose medium (Amemura et al., 1974). Two microliters of 0.3% H_2O_2 were added to a single colony and the number of O_2 gas bubbles produced was counted using a Carl Zeiss binocular microscope. From the colonies growing in the replica plates, dilution series were made and plated in Harada medium to determine cell viability. The assay was conducted five times independently, analyzing each time 10 colonies per strain. Statistical analysis was performed using Student's *t*-test and a level of $P<0.05$ was accepted as significant.

We previously reported that NET30-M1024 produced lower amounts of EPS (73% of the NET30-wt) when grown in YEM medium (mannitol $10\,g\,l^{-1}$ as carbon source) and, when in symbiosis with peanut, induces a lower number of nodulated plants (92%) and of nodule per plant (50%) (Morgante et al., 2005). When NET30-M1024 was grown in Harada medium (glucose $2.5\,g\,l^{-1}$ as carbon source), the EPS content was low, about 46% of NET30-wt content. In this condition, empty non-fixing nodules were observed with a transmission electron microscope.

In this work, the symbiotic properties of NET30-M1024 growing in MM1 medium (fructose $1\,g\,l^{-1}$) were determined. Under this condition, the mutant produced 57% of NET30-wt EPS content. As it was expected, deeper alterations were observed in the symbiotic phenotype of this mutant when lower amounts of EPS were produced (Table 1). Only 25% of plants inoculated with NET30-M1024 were nodulated and these plants showed chlorosis. The number of nodules induced by NET30-M1024 was 5% of those induced by NET30-wt and their dry weight was highly reduced (63%). A significant reduction in the shoot dry weight and total nitrogen content (24% and 27%, respectively) were also found in plants inoculated with the mutant strain. The control uninoculated plants, carried no nodules and showed about the same dry weight and nitrogen content that plants inoculated with NET30-M1024. These results show that the alterations in symbiotic behavior of NET30-M1024 are correlated with the EPS

Table 1
Symbiotic phenotype of the NET30-wt and NET30-M1024 strains

Bacteria	Symbiotic parameters			Agronomic parameters	
	Nodulated plants (%)	No. of nodules per plant	Nodule dry weight (mg/plant)	Shoot dry weight (g/plant)	Nitrogen content (mg/plant)
NET30-wt	100[a]	42±10[a]	13.5±1.1[a]	0.79±0.02[a]	28.98±0.98[a]
NET30-M1024	25[b]	2±1[b]	5.0±0.9[b]	0.60±0.08[b]	21.34±1.71[b]
Uninoculated	0	0	0	0.65±0.08[b]	19.98±1.21[b]

Data are the means ± SE of eight independent determinations.
Values followed by different letters in a column were significantly different ($P< 0.05$) using Student *t*-test.