

Biochemical mechanisms of induced resistance by rhizobial lipopolysaccharide in pea against crenate broomrape

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Received: 28 May 2015 / Accepted: 18 September 2015 / Published online: 5 October 2015
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Abstract Lipopolysaccharides (LPS) isolated from rhizobia have been demonstrated as inducer of systemic resistance against plant diseases. The role of bacterial LPS in induced resistance in plant has not been extensively reported. The capacity of *Rhizobium leguminosarum* Mabrouk et al. 2007 strain P.SOM to reduce parasitism of pea by *Orobanche crenata* Mabrouk et al. 2007 was previously demonstrated under greenhouse and field trials. The objective of this study was to examine the capacity of LPS isolated from strain P.SOM to induce resistance in pea against crenate broomrape, with more emphasis on the biochemical mechanisms of host plant defense reaction induced by LPS. Root chamber experiments using Petri dishes revealed that LPS reduce broomrape seed germination and restricted the broomrape attachment to the host root as well as retarded tubercle formation and development. In pot experiments, pea roots treatment with LPS reduced the total number of broomrape by up to 95 %. No biomass reduction of pea was observed after application of LPS. Broomrape necrosis was observed both before and after parasite attachment to treated pea roots in Petri dishes and pots experiments. Concomitantly, reduction in infection was accompanied by enhanced phenylalanine ammonia lyase, peroxidase, and polyphenoloxidase activities. As a consequence, the derived products, phenolics, and pisatin

accumulated in response to LPS and conferred mechanical and chemical barriers to the invading parasite. Our findings indicate an important role for LPS as a direct inducer of defenses in pea against broomrape.

Keywords *Orobanche crenata* · Pea · *Rhizobium leguminosarum*

Introduction

The use of antagonistic rhizobacteria in biological control showed that these microorganisms can function not only directly by competition and antibiosis, but also indirectly by inducing systemic resistance in the plant toward soil-borne pathogens (van Peer et al. 1991; Liu et al. 1995; Hasky-Günther et al. 1998). Induced resistance using rhizobacteria has been demonstrated against many microorganisms such as fungi, bacteria, and viruses in Arabidopsis, bean, cucumber, and radish. Furthermore, some *Rhizobium* strains can reduce pea and chickpea parasitism by *Orobanche crenata* and *Orobanche foetida* (Mabrouk et al. 2007; Hemissi et al. 2013; Mabrouk and Belhadj 2014). Indeed, bacterial compounds involved in induced plant defense mechanisms are highly variable depending on bacterial strain and pathosystems. For example, many bacterial strains produce salicylic acid (SA), and exogenously applied SA can induce resistance in many plant species (De Meyer and Höfte 1998). Treatments of tomato and radish plants with lipopolysaccharides (LPS) of non-pathogenic pseudomonads induced resistance against pathogenic bacteria (Müller et al. 1998). Similarly, LPS of *Pseudomonas fluorescens* strain WCS 417 have induced systemic resistance (ISR) in carnation against *Fusarium* wilt caused by *F. oxysporum* f. sp. *dianthi* (Van Peer and

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Schippers 1992). In the case of *Rhizobium etli*, LPS have been implicated in triggering the ISR in potato against cyst nematode *Globodera pallida* (Reitz et al. 2000, 2002).

Previously we have demonstrated that living and heat-killed cells of *Rhizobium leguminosarum* P.SOM induced in pea roots systemic resistance against *O. crenata* infection (Mabrouk et al. 2007). Rhizobia produce several surface polysaccharides that are critical for attachment and biofilm formation; some of these polysaccharides are specific for their growth on root hairs and can considerably enhance their ability to infect their host legumes (Downie 2010). LPS belong to the cocktail of microbe derived molecules, which plant cells are exposed to upon bacterial infection (Boller and Felix 2009; Zhang and Zhou 2010; Segonzac and Zipfel 2011). LPS are major constituent of the Gram-negative outer membrane and are released from the bacterial cell wall into the plant apoplast through shedding by living cells or dissolution of dying or dead cells. In addition, LPS are important component of outer membrane vesicles, released from bacterial membranes (Sidhu et al. 2008). When plant cells perceive LPS, it can lead to the triggering of defense responses (Gerber et al. 2004; Zeidler et al. 2004) or to the priming of the plant (Newman et al. 2007; Madala et al. 2012). For the biological activity, kutkowska et al. (2011) demonstrated that polysaccharide obtained by gel chromatography on Bio-Gel P-4 of the high molecular mass material from Rt120 had a toxic effect on tumor HeLa cells but was inactive against the normal human skin fibroblast cell line.

This work aims to evaluate the effect of LPS purified from *R. leguminosarum* P.SOM strain on pea parasitism with broomrape and to characterize the induced resistance in pea by LPS.

Materials and methods

Bacterial strain and growth conditions

Rhizobium leguminosarum strain P.SOM used in this study was isolated from pea roots harvested from *Orobanche*-free crops. This strain was cultured on a yeast extract mannitol medium at 28 °C (Mabrouk et al. 2007).

Isolation of rhizobial lipopolysaccharide

Lipopolysaccharide of *R. leguminosarum* strain P.SOM was extracted from cells grown in 1 l of YEM broth by the hot phenol-water method described by Westphal and Jann (1965). After removal of exopolysaccharides by washing three times with 0.5 M NaCl containing 5 mM EDTA, the bacterial pellet was then lyophilized and

suspended in 50 ml of buffer L containing 50 mM sodium phosphate buffer [pH 7.0], 5 mM EDTA and 0.05 % sodium azide. To remove nucleic acids, the bacterial extract was then treated with DNase and RNase. Remaining protein was digested overnight by incubation with proteinase K. Final LPS extracts were dialyzed (12,000 Da; Serva) for 4 days against demineralized water to remove traces of phenol and again lyophilized. Analysis of LPS patterns by SDS polyacrylamide gel electrophoresis on 15 % gels followed by silver-periodate staining confirmed the presence of LPS constructs in the LPS extract. The LPS of *R. leguminosarum* P.SOM separated into seven bands. The LPS clearly possessed characteristic patterns of *Rhizobium* spp, and the proteins are not detectable in the LPS extracts.

Evaluation of LPS as resistance inducers against broomrape (*Orobanche crenata*): Co-culture using Petri dishes experiments

Co-cultures were performed as described by Labrousse et al. (2001). Pea seedlings were transferred to Petri dishes (120 × 120 × 17 mm, Greiner), placed on fiber filter paper (MN 85/90, 12.5 cm diameter, Macherey-Nagel). Roots were spread between the dish cover and fiber filter paper. One perforation was made on upper border of the Petri dish to hold the pea shoot out, and two perforations were made on the lower border to allow plant root feeding in culture medium. A 1-cm-thick rock-wool layer (Master from Grodan, Town) was placed under pea roots on the other side of the glass fiber filter paper. Petri dishes were closed, covered with aluminum foil, and placed vertically in a sterile polypropylene tray containing sterile solution of Coïc neutrophile nutrient, and the culture maintained at 21 °C under a 16-h photoperiod. After 15 days of culture, 3 ml of rhizobial LPS suspensions (1 mg/ml) was added to the roots. In addition, about 100 preconditioned seeds of *O. crenata* were placed regularly at 1–2 mm from roots. Broomrape seed germination was evaluated 15, 30, and 45 days after treatment with rhizobial lipopolysaccharide using a binocular microscope. Four areas per Petri dish were observed, and the number of germinated seeds is counted and expressed as percentage of total seeds. Germination rates were expressed by taking account of the viability of the seed lot used for experiments (70 %). By 30 and 45 days after treatment (DAT), necrotic-germinated seeds that did not succeed in attaching to host roots in the previously selected areas of Petri dishes were counted and expressed as the percentage of total germinated seeds and numbers of necrotic tubercles per plant are recorded. Non-treated pea seedlings growing in contact with *Orobanche* served as controls.

Pot experiments

LPS were tested in a pot experiment with five replicates per treatment. Pea seedlings were transferred to plastic pots (0.5 l) containing a sterilized mixture of local field soil and sand (1:1, v/v) and then either left as such or contaminated with *O. crenata* seeds (5 mg/pot). Three sets of pot cultures were managed simultaneously: (i) pea grown in *Orobanche*-free soil, (ii) pea grown in infested soil, and (iii) pea grown in infested soil treated with LPS isolated from P.SOM strain (inducer of defense). Each pea plant was treated with 3 ml of suspensions of LPS at concentration 1 mg/ml. Plants were irrigated weekly with water. The impact of rhizobial LPS on pea infection by *O. crenata* was estimated at 70-day-old cultures. Pea roots were gently harvested, washed with water, total number of tubercles per plant, the necrotic tubercle as the percentage of total fixed tubercles, and the dry matter of *Orobanche* were recorded.

Defense enzymes assays

Procedures were carried out as described by Mabrouk et al. (2007) for the peroxidase (POX) and polyphenoloxidase (PPO) assays. Pea roots were ground in liquid nitrogen. The frozen powder was added to the extraction buffer (ratio 1:3, wt/v) containing 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 7), 1 % (v/v) Triton X-100, and 2 % (wt/v) insoluble polyvinylpyrrolidone. The mixture was homogenized and centrifuged for 20 min at 10,000 g (4 °C). The supernatant was used immediately for total protein quantification (Bradford 1976) and enzyme assays.

Soluble POX activity was assayed spectrophotometrically at 470 nm in a reaction medium containing 9 mM gallicol, 1 mM hydrogen peroxide, and crude enzyme extract. POX activity was estimated at 30 °C and expressed as U mg^{-1} protein (U: $\mu\text{mole tetragallicol produced per minute}$).

PPO activity was measured spectrophotometrically at 25 °C using catechol as substrate. Initially, 200 μl of crude extract was mixed with 700 μl of phosphate buffer 0.1 M pH 7, and reaction was started by adding 100 μl 0.2 M catechol. The enzyme capacity was calculated from the initial rate of the A_{420} increase and expressed as $\text{DO min}^{-1} \text{mg protein}^{-1}$.

Phenylalanine ammonia lyase (PAL) activity was determined spectrophotometrically at 290 nm by measuring the amount of cinnamic acid formed after incubation of the crude enzyme with L-phenylalanine for a fixed time. The reaction mixture containing 1.4 ml of borate buffer (100 mM, pH 8.8), 0.6 ml of L-phenylalanine (100 mM), and 0.3 ml of the crude enzyme. Following 2 h of incubation at 40 °C, the reaction was stopped by the addition of 0.05 ml of 5 M HCl. Activity was expressed as microgram cinnamic acid produced per hour per milligram proteins.

Total soluble phenolic content measurements

Pea roots (0.5 g FW) were ground in liquid nitrogen using a pestle and mortar. Frozen powders were extracted three times with 5 ml MeOH/water (80/20 v/v) at 4 °C under continuous stirring. The combined homogenates were centrifuged (4000 g, 10 min). Total soluble phenolic content was measured spectrophotometrically (A_{760}) from the supernatant using the Folin–Ciocalteu reagent method (Waterman and Mole 1994). The reaction mixture containing 790 μl of distilled water, 10 μl of sample, and 50 μl of Folin–Ciocalteu reagent. After 1 min, 150 μl of aqueous sodium carbonate (20 %) was added, and the mixture was vortexed and allowed to stand at room temperature with exclusion of light for 30 min. The total phenol concentration was calculated from the calibration curve, using catechin as standard, and the results were expressed as microgram of catechin equivalents per gram FW.

Pisatin content measurements

Pisatin was extracted from freshly harvested roots (0.5 g FW) by shaking tissue gently in 5 ml hexane for 2 days at room temperature. The extract was vacuum evaporated at 35 °C, and then dissolved in 3 ml ethanol. Pisatin was estimated spectrophotometrically as described by Novak et al. (2004) from the specific pisatin A_{307} value of the ethanolic extract (molar extinction coefficient: $10^{3.86}$) following subtraction of the baseline A_{400} value.

Statistical analysis

In all the experiments, 10 plants were grown per treatment. Consequently, the data are means \pm confidence limits ($n = 10$, $\alpha = 0.05$ Student's *t* test). Data were analyzed by multifactorial analysis of variance (ANOVA, SPSS 13.0 for Windows), and significant differences among treatments were considered at the $P < 0.05$ level.

Results

Effect of LPS on pea parasitism by broomrape

Effect of rhizobial LPS on underground stages of O. crenata

Treatment with the LPS extract (3 mg/plant), resulted in significant decrease in germination of *O. crenata* seeds (up to 50 %) and none of the tubercle formed on pea roots at 45 DAT (Table 1). At lowest concentration (1.5 mg/plant), the LPS caused also reduction in broomrape seeds germination by 10 and 30 % respectively at 30 and 45 DAT

Table 1 *Orobanche crenata* germination and tubercles formation in co-culture experiments on pea roots in relation to rhizobial polysaccharides (LPS) treatments

Treatments	30 DAT				45 DAT			
	<i>O. crenata</i> seeds germination (%)	Necrotic-germinated seeds (% of total germinated seeds)	Number of tubercles/plant	Number of tubercles/plant	<i>O. crenata</i> seeds germination (%)	Necrotic-germinated seeds (% of total germinated seeds)	Number of tubercles/plant	Necrotic tubercles (%)
Pea	53.45 ± 7.98	0	28.33 ± 3.23	48.15 ± 4.98	59.66 ± 6.66	2.88 ± 0.42	48.15 ± 4.98	0
Pea + LPS (1.5 mg/plant)	48.33 ± 4.87	0	5.45 ± 0.55	9.48 ± 1.25	42.85 ± 2.65	22.87 ± 4.68	9.48 ± 1.25	25.23 ± 1.45
Pea + LPS (3 mg/plant)	38.66 ± 3.87	0	0	0	28.35 ± 4.65	52.87 ± 4.68	0	0

(Table 1). LPS can act directly on *Orobanche* seed germination or indirectly by inducing metabolic change in pea roots. To check these hypotheses, the effect of LPS was determined on *Orobanche* seed germination in the presence of synthetic germination stimulant (GR24). The LPS displayed no effect on germination levels when stimulated by GR24 (Table 2). In contrast, treatment of pea roots by LPS decreased broomrape seed germination rates (Table 1), suggesting that LPS can induce accumulation of germination inhibitors. Changes in some *O. crenata* germinated seeds started to be visible on day 25. Radicles turned from colorless and translucent to brownish, elongation ceased, and *Orobanche* development stopped as it describes previously by Mabrouk et al. (2007). Some disease symptoms on early development stages were observed in the case of treated pea with LPS at low concentration. The proportion of necrotic tubercles increased with time. Forty-five days after transplanting the highest percentage was observed on pea treated with LPS (Table 1). Healthy tubercle developed on the host roots within 14 day attachment. In contrast, when *O. crenata* attacked the pea roots treated with LPS, it died at early stage of tubercle development. Our results showed that LPS inhibited pea parasitism by broomrape at two concentrations tested, but, at 3 mg/plant, LPS protects better the plant against broomrape host. This protection was characterized by no or low number of tubercles attached on pea roots observed in Petri dishes and pots experiments, respectively.

Effect of rhizobial LPS on O. crenata development in pots

In the pot experiments, pea roots treated with LPS (3 mg/pot) resulted in decrease of the number of tubercles on pea roots (Table 3). The total *Orobanche* dry matter per pot was significantly reduced by 75 % in pea roots treated with LPS. Obviously, when plants grew in *Orobanche*-infested soil in pot experiments, pea roots treated with the LPS extract carried few broomrape tubercles by 70 DAT in comparison with non-treated peas (Table 3). As observed in Petri dishes (Table 1), a relatively high percentage of these tubercles turned brown.

Effect of rhizobial LPS on defense enzymes activities and toxic compounds accumulation in pea roots

Enzyme activities were verified by 45 DAT in both infected and treated peas (Fig. 1) and compared with activity levels of the control plants including healthy and infected non-treated peas. In pea plants treated with LPS, the expression of induced resistance was associated with enhanced enzyme activities.

Table 2 Effect of LPS on *Orobanche* seed germination in vitro

Treatment	Water	GR24	GR24 +LPS
<i>Orobanche</i> seed germination (%)	0	80.45 ± 2.58	79.87 ± 1.89

Table 3 Impact of root treatment by rhizobial LPS on tubercles formation and *Orobanche* dry matter in pot experiments

Treatments	Tubercles formation		<i>Orobanche</i> dry matter
	Number of tubercles/plant	Necrotic tubercles (% of total tubercles)	<i>Orobanche</i> DW (g)
Pea	45.23 ± 3.25	0	1.54 ± 0.21
Pea + LPS (1.5 mg/plant)	10.45 ± 1.55	20.50 ± 2.75	0.45 ± 0.11
Pea + LPS (3 mg/plant)	2.36 ± 0.78	50.45 ± 2.33	0.12 ± 0.05

POX and PPO activities increased gradually from 7 to 35 DAT when peas were treated singly by LPS (Fig. 1a, b). A similar pattern was observed in roots treated by LPS and concomitantly infected by the parasitic weed. On the other hand, low and relatively constant activity occurred in healthy and infected peas when not treated by LPS.

PAL activity remained unchanged by 35 DAT at a low value in healthy pea roots (Fig. 1c). Furthermore, infection by the *Orobanche* did not significantly affect PAL activity in roots. In contrast, pea treatment with LPS induced an obvious threefold rise in PAL activity of infested peas which attained a maximal value at 15 DAT when neither parasite attachment to pea roots.

Total soluble phenolic compounds accumulated later from 28 to 35 DAT in pea treated by the rhizobial LPS (Fig. 2a). A similar pattern was observed when the treated pea by LPS was concomitantly infected by *Orobanche*. On the other hand, healthy pea and plants only infected by *Orobanche* exhibited low relative amounts of total soluble phenolics.

Pisatin amounts were constantly close to a basal value (20 $\mu\text{mol g}^{-1}$ FW) in roots not treated by LPS and roots only infected by *Orobanche* (Fig. 2b). In contrast, treatment by LPS induced an obvious rise in pisatin content that could be detected from 15 DAT. Although high pisatin content remained at 21 DAT, accumulation decreased significantly from 21 to 35 DAT. Nevertheless, the remaining amount of pisatin by 35 DAT was significantly higher than in peas that had not been treated by LPS. This pattern was similar in roots both treated with LPS and infected by *Orobanche*.

Discussion

Plant beneficial microorganisms are increasingly being used in sustainable agriculture. Several modes of action have been described, including competition for nutrients,

antibiosis, induced resistance, mycoparasitism, plant growth promotion, and rhizosphere colonization capability (Hassanein et al. 2006; Siddiqui and Akhtar 2007; Bailey et al. 2008). Many studies demonstrated that specific rhizobacteria reduce plant infection by various parasitic plant such as *O. crenata* and *O. foetida* (Mabrouk et al. 2007; Hemissi et al. 2013; Mabrouk and Belhadj 2014). In our previously studies, it was shown that the *R. leguminosarum* P.SOM strain reduced pea roots infection by *O. crenata* indirectly by inducing systemic resistance (Mabrouk et al. 2007). Since the plant defense capacity in pea roots was enhanced by both living and heat-killed cells of this strain, it was concluded that heat stable surface structures such as LPS act as inducing agents (Mabrouk et al. 2007).

We demonstrated that pea roots treatment with LPS extract significantly decreases their susceptibility to the parasite *O. crenata* indicating the implication of these compounds in induced resistance. The effect direct on broomrape seeds germination and development can be excluded, because LPS did not affect the germination rate of seeds induced by GR24. Induced resistance in treated peas was shown to occur throughout the infection process. Indeed, induced resistance was expressed at different developmental stages of *Orobanche* including germination, radicle growth, parasite attachment to pea roots, and finally tubercle growth on host roots. It has been shown that LPS of *P. fluorescens* strain WCS 417 have ISR in carnation against *Fusarium* wilt caused by *F. oxysporum* f. sp. *dianthi* (van Peer and Schippers 1992). Similarly, LPS of *P. fluorescens* strains WCS 374 and WCS 417 have ISR in radish against *F. oxysporum* f. sp. *raphani* (Leeman et al. 1995). They also established that mutant of *P. fluorescens* strain WCS 417, lacking the O-antigen side chain of the LPS, has not induced resistance in radish indicating that the O-antigen side chain of the LPS might have served as a signal or trigger in the induction of defense mechanism in plants. In contrast, LPS of *P. putida* strain WCS 358 having O-antigen side chain do not induce systemic resistance in

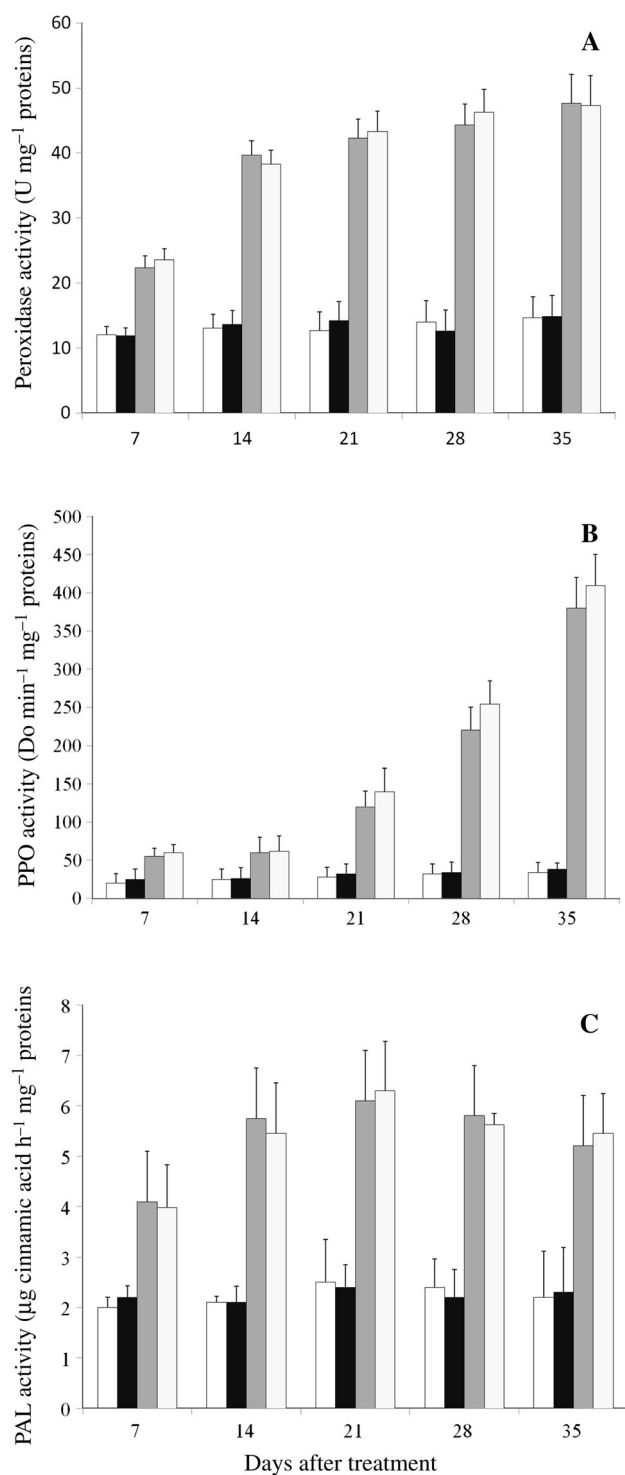


Fig. 1 Impact of pea root treatment with LPS on the peroxidase (a), PPO (b) and PAL (c) activities extracted from pea roots infected by *Orobanche crenata*. Activities were measured on 7, 14, 21, 28, and 35 DAT. Controls were performed with non-treated and healthy peas (white vertical box), and non-treated and infested peas (black vertical box), peas treated only with LPS (gray vertical box), peas treated with LPS and infested (white vertical box)

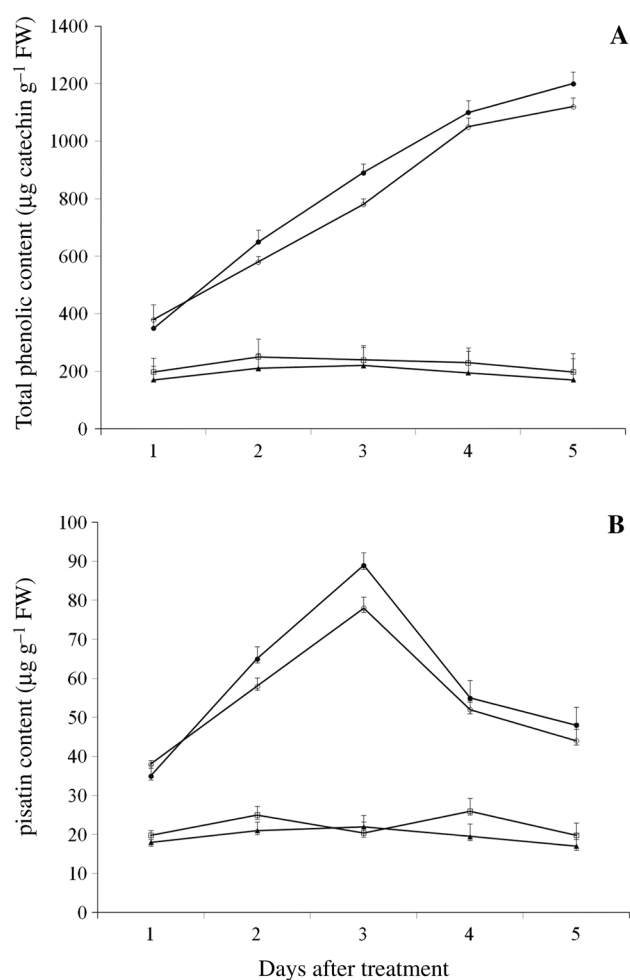


Fig. 2 Changes in total phenolic (a) and pisatin (b) contents in pea roots following treatment with rhizobial LPS and infection by *Orobanche crenata*. Activities were measured at 7, 14, 21, 28, and 35 DAI in pea treated with LPS and concomitantly infected by *O. crenata* (black circle). Controls were performed with healthy pea (black triangle), pea singly treated with LPS (white circle) or singly infected by *O. crenata* (white square)

radish. In another study, LPS of WCS 417r and mutant of WCS 417r lacking O-antigen side chain of LPS elicit defense mechanism in *Arabidopsis* (Van Wees et al. 1997).

The obvious increase in the total phenolics and pisatin levels in the infected than healthy pea roots that treated with LPS, indicates their effectiveness in inducing resistance. In this regards, soluble phenolics and pisatin have been shown to accumulate in plant roots that play important role in the parasitic infection process and host resistance (Sahm et al. 1995; Mabrouk et al. 2007).

Activity of all defense enzyme was significantly affected by the treatment by LPS. Phenylalanine ammonia lyase (PAL) is the first enzyme of phenylpropanoid pathway and

plays a significant role in regulating the phenolics biosynthesis in plants (Daayf et al. 1997). Induction of PAL is correlated with increased resistance to broomrape (Mabrouk et al. 2007). POXs can polymerize polysaccharides and polyphenols to produce stable vascular occluding gels (Crews et al. 2003). Antioxidant enzyme polyphenol oxidase (PPO) is a copper-containing ubiquitous enzyme reported to catalyze oxidation of phenolics to more toxic quinones. PPO-generated quinones and ROS can play an array of defense-related functions simultaneously.

In this study, LPS-treated peas displayed an enhanced POX and PPO activities, in addition to a constantly high PAL activity in comparison with non-inoculated peas. This was observed from 15 DAT when broomrape attachment did not occur. Consequently, we hypothesize that these enzymes could be involved in pea resistance to crenate broomrape induced by LPS application during early and later stages of infection. Consequently, three enzymes could be implicated in broomrape avoidance of inoculated pea by preventing parasite penetration of the host root or by lowering nutrient fluxes toward the parasite when connection succeeds. The present results are in agreement with others studies describing another pathosystems. LPS of strain RS83 plays a role in the induction of early defensive-related enzymes in lettuce against soft rot disease caused by *Pectobacterium catovorum* subsp. *catovorum* (Jetiyanon and Plianbangchang 2013).

We conclude that application of LPS of the *Rhizobium* strain P.SOM can protect pea against *O. crenata*, by reducing host root active exudation and by preventing parasite attachment and growth of installed tubercles. High level of the defense enzymes, POX, PPO, and PAL by LPS application may contribute collectively to induced resistance in pea against broomrape. Finally, economic analysis remains to be performed to verify the feasibility of LPS treatments at larger scales.

Acknowledgments This work is funded by the project “PHC Uti-que 13G0925” managed by the Joint University Committee of Cooperation (Tunisia-France).

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