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Defence-Related Enzymes in Soybean Resistance to Target Spot

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Abstract

Target spot, caused by the fungus *Corynespora cassiicola*, has become a serious foliar disease in soybean production in the Brazilian Cerrado. Information in the literature regarding the biochemical defence responses of soybean to *C. cassiicola* infection is rare. Therefore, the objective of this study was to determine the biochemical features associated with soybean resistance to target spot. The activities of chitinases (CHI), β-1-3-glucanases (GLU), phenylalanine ammonia-lyases (PAL), peroxidases (POX), polyphenol oxidases (PPO) and lipoxygenases (LOX), as well as the concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives, were determined in soybean leaves from both a resistant (FUNDACEP 59) and a susceptible (TMG 132) cultivar. The target spot severity, number of lesions per cm^2 of leaflet and area under the disease progress curve were significantly lower for plants from cv. FUNDACEP 59 compared to plants from cv. TMG 132. The GLU, CHI, PAL, POX and PPO activities and the concentration of LTGA derivatives increased significantly, whereas LOX activity decreased significantly on the leaves infected by *C. cassiicola*. Inoculated plants from cv. FUNDACEP 59 showed a higher PPO activity and concentrations of TSP and LTGA derivatives at 4 and 6 days after inoculation compared to plants from cv. TMG 132. In conclusion, the results of this study demonstrated that the defence-related enzyme activities increased upon *C. cassiicola* infection, regardless of the basal level of resistance of the cultivar studied. The increases in PPO activity and concentrations of TSP and LTGA derivatives, but lower LOX activity, at early stages of *C. cassiicola* infection were highly associated with soybean resistance to target spot.

Introduction

Soybean [*Glycine max* (L.) Merrill] is considered to be one of the most important crops grown in Brazil and other countries worldwide (Godoy et al. 2012). However, the occurrence of many foliar diseases on soybean has caused significant yield losses (Yorinori 1997; Sinclair 1999). Target spot, caused by the fungus *Corynespora cassiicola* (Berk & MA Curtis) CT Wei., has become an important foliar disease affecting soybean production in Brazil. A symptom of target spot on leaves is the occurrence of small lesions with a very well developed yellow halo, which can reach two inches in diameter, become circular and brownish and may coalesce as they expand (Sinclair 1999; Almeida et al. 2005). When susceptible soybean cultivars are grown under favourable climatic conditions (*e.g.* intense rainfall and high temperatures), premature defoliation can occur (Godoy et al. 2012; Teramoto et al. 2013). The fungus is also able to cause rot in roots and pods and lesions on stems, and it can eventually infect the seeds (Sinclair 1999). Seed treatment with fungicides, foliar spray of fungicides and crop rotation are the most used control strategies for target spot management (Almeida et al. 2005). However, due to the absence of resistant cultivars and the occurrence of *C. cassiicola* isolates resistant to fungicides, epidemics of target spot have been frequent in many soybean-growing regions in Brazil (Godoy et al. 2012; Teramoto et al. 2013). As a foliar

and necrotrophic pathogen, *C. cassiicola* completely destroys the leaf cells as a strategy to obtain nutrients, thereby achieving massive growth in the host's tissue (Onesirosan et al. 1975b; Lamotte et al. 2007). The diffusion of many hydrolytic enzymes and non-host selective toxins into the healthy leaf tissues further increases the cellular damage by *C. cassiicola* infection (Onesirosan et al. 1975b; Lamotte et al. 2007).

Plants respond to pathogen infections through many different defence mechanisms, which include the expression of several defence-related genes, the synthesis of antimicrobial compounds such as phenolics and phytoalexins, the production of reactive oxygen species and tissue lignification (Lozovaya et al. 2004; Hückelhoven 2007; Upchurch and Ramirez 2010). The plant's ability to reduce fungal colonization is most likely dependent on the combination of different mechanisms of defence, which seem to greatly change according to the host–pathogen interaction (Hammond-Kosack and Jones 1996; Hammond-Kosack and Parker 2003).

In the soybean–*Phakopsora pachyrhizi* interaction, an early increase in the transcript levels of some defence genes occurred on plants from the accession PI230970 carrying the *Rpp2* resistance gene, in comparison with a susceptible cultivar (Van de Mortel et al. 2007). The phenylalanine ammonia-lyase (PAL) was of great importance on the leaves of a resistant rice cultivar to reduce the symptoms of the *Rice strip virus* (in contrast to a susceptible cultivar) (Hao et al. 2011). Compared with a susceptible cultivar, the genes coding for the enzymes PAL and chalcone synthase and for the pathogenesis-related proteins PR1 and PR5 were up-regulated in the soybean roots from a resistant cultivar infected by *Fusarium solani* f.sp. *glycines* (Iqbal et al. 2005). The pattern of gene expression in soybean roots infected by *Phytophthora sojae* showed that lipoxygenase and polyphenol oxidase were strongly down-regulated during the course of the pathogen infection (Moy et al. 2004).

Information in the literature regarding the biochemical defence responses of soybean plants to *C. cassiicola* infection is rare. Therefore, the objective this study was to determine the biochemical features associated with soybean resistance to target spot.

Materials and Methods

soybean growth

A total of ten soybean seeds from cvs. TMG 132 and FUNDACEP 59 (susceptible and partially resistant to target spot (Godoy et al. 2012; Pitol et al. 2011), respectively) were sown into 2-L plastic pots (Ecovaso, Jaguariúna, SP, Brazil) containing 2 kg of Tropstrato® (Vida Verde, Mogi Mirim, SP, Brazil) substrate composed of a 1:1:1 mixture of pine bark, peat and expanded vermiculite. Five days after seedling emergence, each pot was thinned to two seedlings, which were fertilized weekly with 50 ml of a nutrient solution prepared using deionized water and containing 40 mM KNO_3, 10 mM $NH_4H_2PO_4$, 10 mM $MgSO_4.7H_2O$, 15 mM $Ca(NO_3)_2.4H_2O$, 2.4 mM $ZnSO_4.7H_2O$, 3 mM H_3BO_3, 10 mM K_2SO_4, 3.3 mM CH_4N_2O and 7.5 mM $NH_4H_2SO_4$ (Dallagnol et al. 2012). Plants were also watered with deionized water as needed. The plants were maintained in a greenhouse (relative humidity 65 ± 5% and temperature 30 ± 5°C) during the experiments.

Inoculation procedure

A pathogenic isolate of *C. cassiicola*, obtained from symptomatic soybean leaves plants collected in the municipality of Rio Verde, Goiás State, Brazil, was used to inoculate the plants. The *C. cassiicola* was isolated from the diseased leaves, and the morphological identification of the conidia was performed. The isolate of *C. cassiicola* was preserved using Castellani's method (Dhingra and Sinclair 1995). At 14 days before inoculation, plugs of potato dextrose agar medium containing fungal mycelia (preserved by Castellani's method) were placed into Petri dishes containing carrot leaf-pea dextrose agar (CL-PeDA) media. The CL-PeDA media was prepared using 200 mg of carrot leaves, 100 g of fresh peas, 20 g dextrose and 20 g agar. Prior to media preparation, carrot leaves and peas were placed in a blender to obtain a homogenous mixture, which was then sieved to remove the excess solids. Fragments of fungal mycelia were transferred and homogenously spread onto new Petri dishes. The Petri dishes were placed in a growth chamber at 25°C with a 12-h photoperiod for 4 days. After this period, the fungal colonies were stressed using a Drigalski spatula in a laminar flow chamber, to avoid contamination (Onesirosan et al. 1975a). The plates were then maintained in a growth chamber under continuous white light (40 W lamps alternately distributed to provide a light intensity of 165.3 μmol/s/m^2) for 6 days until the production of conidia. Conidia were carefully removed from the Petri dishes with a soft-bristle brush using water containing gelatin (1% w/v). Plants were grown for 45 days (V9 growth stage) (Fehr et al. 1971) and then inoculated with a conidial suspension of *C. cassiicola* (5×10^4 conidia per ml). A total of 20 ml of conidial suspension was

applied as a fine mist using a VL Airbrush atomizer (Paasche Airbrush Co., Chicago, IL, USA) to both adaxial and abaxial leaf surfaces of each plant. After inoculation, plants were maintained in a plastic mist growth chamber (MGC) inside a greenhouse for the duration of the experiments. The MGC was constructed of wood (2 m wide, 1.5 m high and 5 m long) and covered with transparent plastic (100 μm thick). The maximum natural photon flux density at the plant canopy height was approximately 700 μmol/m^2/s^1, and the temperatures were 25 ± 2°C (day) and 20 ± 2°C (night). The relative humidity was maintained at 90 ± 5% using a misting system where nozzles (model NEB-100, KGF Co., São Paulo, Brazil) sprayed a mist every 30 min for 15 s above the plant canopy. The temperature and relative humidity were measured with a thermohygrograph (TH-508; Impac, São Paulo, Brazil).

Disease assessment

The target spot severity (TSS) was evaluated on the 8th trifoliate leaf from plants of each replication and treatment at 4, 6, 8 and 10 days after inoculation (dai) using a standard area diagram set (Soares et al. 2009). Data from TSS were used to calculate the area under disease progress curve (AUDPC) according to the method of Shaner and Finney (1977). At 10 dai, the number of lesions (NL) per cm^2 of leaf area was counted at five randomized places on the 8th trifoliate leaves from plants of each replicate and cultivar with the aid of a hand-held microscope (15×).

Biochemical assays

For all biochemical assays, the 6th, 7th, 8th and 9th trifoliate leaves (from base to the top) of plants from the replications of each treatment were collected at 4, 6, 8 and 10 dai. Leaf samples were held in liquid nitrogen during sampling and then stored at −80°C until further analysis.

Determination of defence enzymes activities

To determine the activities of phenylalanine ammonia-lyases, β-1,3-glucanases (GLU), chitinases (CHI), peroxidases (POX) and polyphenol oxidases (PPO), a total of 200 mg of leaf tissue was ground into a fine powder with liquid nitrogen using a mortar and pestle. The fine powder was homogenized in 2 ml of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% (w/v) polyvinylpyrrolidone (PVP). Then, the homogenate was centrifuged at 12 000 *g* for 15 min at 4°C; the supernatant was collected and used to determine the GLU, CHI, PAL, PPO and POX activities. The GLU activity was determined according to the method of Lever (1972). First, 20 μl of the crude enzyme extract was added to a reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and laminarin (1 mg/ml). Next, the reaction mixture was incubated in a ThermoMixer (Eppendorf, Hamburg, Germany) at 45°C for 1 h. Then, 500 μl of the reaction mixture was added to 1.5 mL of dinitrosalicylic acid (DNS) and incubated at 100°C for 15 min. The reaction was stopped using an ice bath until the solution reached 25°C. The amount of reducing sugars released was calculated with a calibration curve using glucose (Sigma-Aldrich, São Paulo, Brazil) as a standard, and the absorbance was measured at 540 nm (Miller 1959). A similar procedure was used for the control samples, but the first incubation was excluded. The CHI activity was determined according to the method of Harman et al. (1993). The reaction was started with the addition of 20 μl of the crude enzyme extract to 1980 μl of a reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and 0.1 mM *p*-nitrophenyl-β-*D*-*N*-*N′*-diacetylchitobiose. Next, the reaction mixture was incubated at 37°C for 2 h, and the reaction was stopped by adding 500 μl of 0.2 M sodium carbonate. The control samples received 500 μl of 0.2 M sodium carbonate immediately after the addition of the crude enzyme extract to the reaction mixture. The final product released by CHI was measured at 410 nm, and the CHI activity was based on the extinction coefficient of 70 mM/cm. The PAL activity was assayed following the method proposed by Guo et al. (2007) with some modifications. First, the reaction was started by adding 100 μl of crude enzyme extract to 0.9 ml of a reaction mixture containing 40 mM sodium borate buffer (pH 8.8) and 20 mM *L*-phenylalanine. The reaction mixture was incubated at 30°C for 1 h. For the control samples, the extract was replaced by borate buffer. The reaction was stopped by adding 50 μl of 6 N HCl. The absorbance of the trans-cinnamic acid derivatives was recorded at 290 nm. The PAL activity was estimated with an extinction coefficient of 10 M/cm (Zucker 1965). The PPO activity was assayed following the colorimetric determination of pyrogallol oxidation according to the method of Kar and Mishra (1976) with some modifications. The reaction was started after the addition of 15 μl of the crude enzyme extract to 985 μl of a reaction mixture containing 25 mM potassium phosphate buffer (pH 6.8) and 20 mM

pyrogallol. Immediately after the reaction was initiated, the absorbance was determined at 420 nm for 1 min at 25°C. The PPO activity was calculated using the extinction coefficient 2.47 mM/cm according to the PPO ionically bound to the cell wall (Chance and Maehley 1955). The POX activity was determined using the same procedure as described for PPO; however, the reaction mixture contained 20 mM hydrogen peroxide.

The lipoxygenase (LOX) activity was assayed according to the method described by Axelrod et al. (1981). The reaction was started after the addition of 10 μl of the crude enzyme extract to 900 μl of 50 mM sodium phosphate buffer (pH 6.8) and 15 μl of 10 mM sodium linoleate substrate. Then, the reaction mixture was incubated at 25°C for 4 min. The absorbance of the product released by the LOX for 1 min was measured in a spectrophotometer at 234 nm. The extinction coefficient of 25 000 M/min was used to calculate the LOX activity.

The enzyme activity was expressed based on protein concentration determined according to the method of Bradford (1976).

Determination of the concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives

A total of 100 mg of leaf tissue was ground into a fine powder with liquid nitrogen using a mortar and pestle and homogenized in 1 ml of a solution containing 80% (v/v) methanol. Next, the crude extract was shaken at 300 rpm at 25°C for 2 h. Subsequently, the mixture was centrifuged at 17 000 ***g*** for 30 min. The methanolic extract was collected and used to determine the TSP concentration, and the pellet was maintained at 20°C to further determine the LTGA derivatives concentration. The TSP concentration was assayed following the methodology proposed by Zieslin and Ben-Zaken (1993) adapted by Rodrigues et al. (2005). The reaction was started after the addition of 150 μl of methanolic extract to 750 μl of 0.2 M Folin-Ciocalteu phenol reagent and incubated at 25°C for 5 min. The next step was the addition of 0.1 M sodium carbonate to the solution, which was maintained at 25°C for 10 min. Afterwards, 1 ml of deionized water was also added to the mixture and the solution was incubated at 25°C for 1 h. The TSP concentration was calculated based on a calibration curve using catechol (Sigma-Aldrich) as a standard, and the absorbance was read at 725 nm. For the determination of the LTGA derivative concentration, the pellet was resuspended in 1.5 ml of deionized water and homogenized and centrifuged at 12 000 ***g*** for 15 min. Afterwards, the supernatant was discarded and the pellet was dried at 65°C for 12 h. The alcohol-insoluble dry residue was used to determine the concentration of LTGA derivatives as described by Barber and Ride (1988). The absorbance of the LTGA derivatives supernatant was read at 280 nm, and the concentration was determined from a calibration curve, using lignin, alkali and 2-hydroxypropyl ether (Sigma-Aldrich) as standards.

Experimental design and data analysis

An experiment consisting of two cultivars (TMG 132 and FUNDACEP 59) was arranged in a completely randomized design with 12 replications to evaluate the TSS and the NL. A 2 × 2 × 4 factorial experiment consisting of two cultivars, non-inoculated and inoculated plants, and four sampling times with four replications was arranged in a completely randomized design to obtain the leaf samples for the biochemical analysis. For the TSS, an ANOVA was used to analyse the 2 × 4 factorial experiment (consisting of two cultivars and four evaluation times). The NL and AUDPC were analysed using a one-way ANOVA with two cultivars. The biochemical variables were analysed using an ANOVA for the 2 × 2 × 4 factorial with two cultivars, two plant inoculations (non-inoculated and inoculated plants) and four sampling times. Each experimental unit consisted of a 2-l plastic pot with two plants. Data from all variables were analysed by analysis of variance (ANOVA), and means from the treatments were compared by *t*-tests ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC, USA). The Pearson linear correlation technique was used to determine the relationships among TSS and the GLU, CHI, PAL, POX and PPO activities as well as the concentrations of TSP and LTGA derivatives.

Results

The cultivar (C) factor was significant for the NL and AUDPC (Table 1). The C and sampling time (ST) factors, as well as their interaction, were significant for TSS (Table 1). The target spot symptoms, characterized by circular brownish lesions with yellow halos, were more evident on the leaflets of plants from cv. TMG 132 than from cv. FUNDACEP 59 (Fig. 1a). The TSS was significantly reduced by 50, 22, 17 and 25% at 4, 6, 8 and 10 dai, respectively, on the leaflets of plants from cv. TMG 132 compared to plants from cv. FUNDACEP 59 (Fig. 1c). The NL and AUDPC were

Table 1 Analysis of variance of the effects of cultivars (C), plant inoculation (PI) and sampling time (ST) on target spot severity (TSS), number of lesions (NL) per cm^2 of leaflet, area under disease progress curve (AUDPC) as well as on the activities of β-1-3-glucanases (GLU), chitinases (CHI), phenylalanine ammonia-lyases (PAL), peroxidases (POX), polyphenol oxidases (PPO) and lipoxygenases (LOX) and on the concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives in soybean leaves from cultivars TMG 132 and FUNDACEP 59 inoculated with *Corynespora cassiicola*

Variables	C^z	PI	ST	C × PI	C × ST	PI × ST	C × PI × ST
TSS	***	–	***	–	***	–	–
NL	***	–	–	–	–	–	–
AUDPC	***	–	–	–	–	–	–
GLU	ns	***	ns	ns	ns	**	ns
CHI	ns	***	**	ns	ns	ns	ns
PAL	ns	***	ns	ns	ns	ns	ns
POX	ns	***	**	ns	ns	**	ns
PPO	ns	***	***	ns	ns	***	ns
LOX	*	***	***	ns	***	***	ns
TSP	ns	***	***	ns	ns	***	ns
LTGA	**	ns	**	ns	ns	ns	ns

^{z}Levels of probability: ns = non-significant, * = 0.05, ** = 0.01 and *** = < 0.001; – = not determined.

significantly reduced by 26 and 16%, respectively, for plants from cv. FUNDACEP 59 in comparison with plants from cv. TMG 132 (Fig. 1c and d).

For GLU and PAL activities, only the factor plant inoculation (PI) was significant (Table 1). The factors PI and ST were significant for CHI, POX and PPO activities and for the TSP concentration. For the LTGA derivative concentration, only the factors C and ST were significant. The factors C, PI and ST were significant for LOX activity. The interaction PI × ST was significant for GLU, POX, PPO and LOX activities and for the TSP concentration. The interaction C × ST was significant for LOX activity (Table 1).

The GLU, CHI, PAL, POX and PPO activities were significantly increased in the inoculated plants from cvs. TMG 32 and FUNDACEP 59 relative to their non-inoculated counterparts from 4 to 10 dai (Fig. 2). Significant difference between inoculated plants from cvs. TMG 132 and FUNDACEP 59 occurred only for PPO at 4 and 6 dai, with higher activities recorded for the latter cultivar (Fig. 2). The LOX activity was significantly decreased in response to *C. cassiicola* infection (Fig. 2). There was no difference between non-inoculated and inoculated plants from cvs. TMG 132 and FUNDACEP 59 for TSP concentration (Fig. 3). The inoculated plants from cvs. TMG 132 and FUNDACEP 59 had LTGA derivatives concentrations significantly higher than the non-inoculated plants regardless of the ST (Fig. 3). For the inoculated plants from cv. FUNDACEP 59, the concentrations of TSP and LTGA derivatives were significantly higher than for plants from cv. TMG 132 at early stages of *C. cassiicola* infection (Fig. 3).

For cv. TMG 132, there were positive correlations of TSS with POX and PPO activities and the concentration of LTGA derivatives; however, TSS was negatively correlated with LOX activity (Table 2). The LOX activity was negatively correlated with the concentration of LTGA derivatives (Table 2). For cv. FUNDACEP 59, there were positive correlations between TSS and POX activities, GLU and PPO activities and between PAL activity and the concentration of LTGA derivatives. The correlations between TSS and LOX activities, between the activities of PAL and PPO and between the activities of POX and LOX were significantly negative (Table 2).

Discussion

Although target spot has become an increasingly important disease in the Cerrado, the most important soybean-growing area in Brazil, information regarding the mechanisms underlying soybean resistance to *C. cassiicola* infection is still rare in the literature. The results from this study provide novel biochemical information of the importance of defence-related enzymes in soybean resistance against target spot. The lower TSS, NL and AUDPC recorded for plants from cv. FUNDACEP 59 compared to plants from cv. TMG 132 confirmed their basal level of resistance to *C. cassiicola* infection as previously reported (Pitol et al. 2011; Godoy et al. 2012). In agreement with previously reports on the importance of defence enzymes for host resistance against pathogen infections (Roulin and Buchala 1995; Li and Steffens 2002; Siranidou et al. 2002; Lozovaya et al. 2004; Sahoo

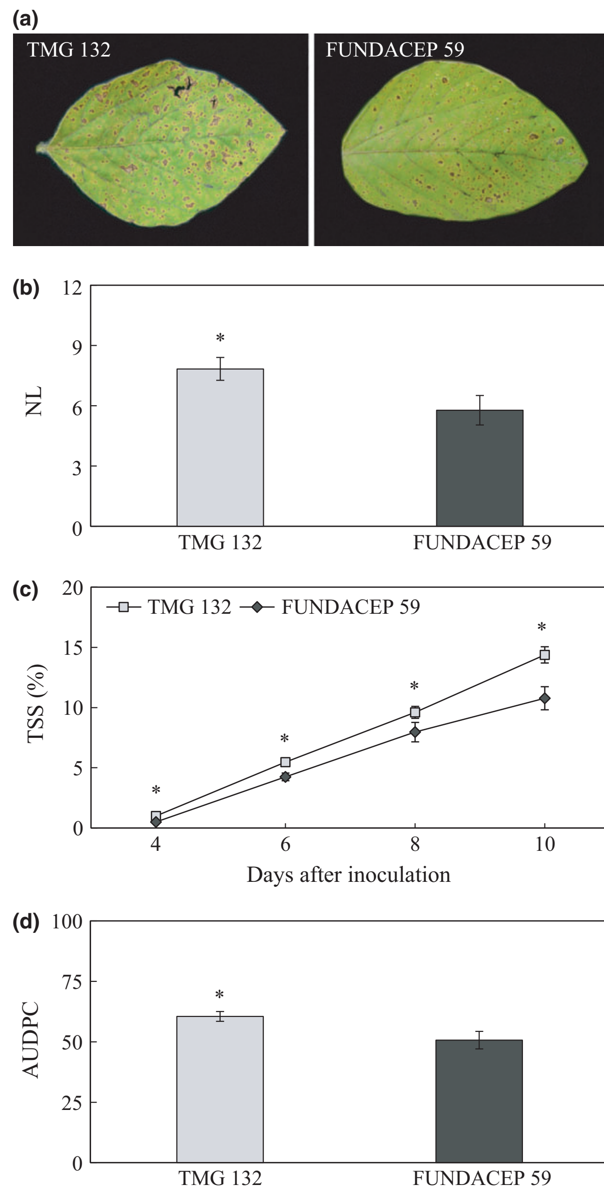


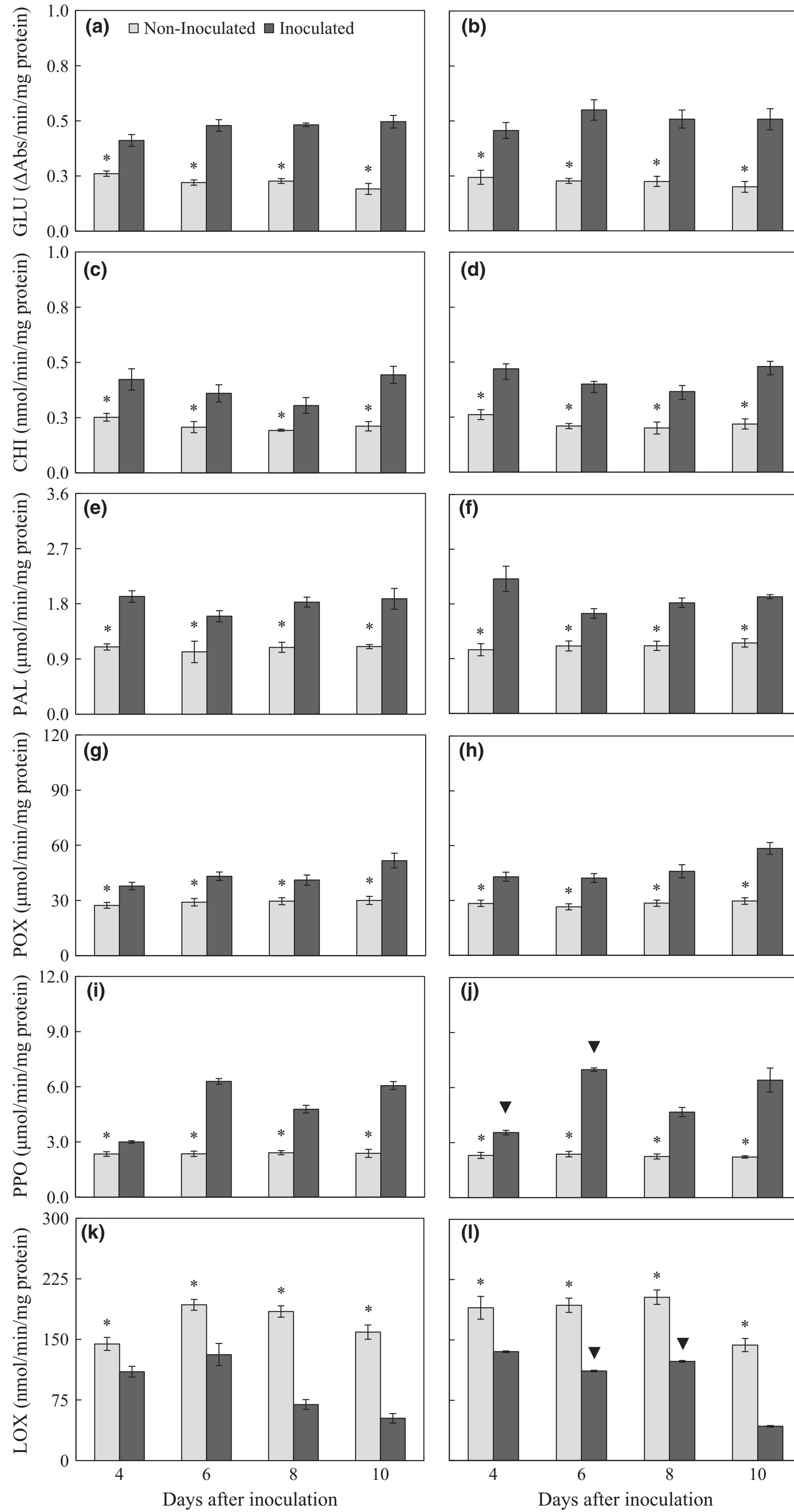
Fig. 1 Symptoms of target spot (a), number of lesions (NL) per cm^2 of leaflet (b), target spot severity (TSS) (c) and area under disease progress curve (AUDPC) (d) in soybean leaves from cultivars TMG 132 and FUNDACEP 59 inoculated with *Corynespora cassiicola*. The means for either NL or AUDPC between the cultivars as well as between the cultivars within each sampling time for TSS followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard errors of the means. Two experiments were conducted with consistent results; the results of one representative experiment are shown.

et al. 2009; Gnanamangai et al. 2011; Leite et al. 2014), the more pronounced increases in the activities of the defence enzymes, as well as increases in the concentrations of TSP and LTGA derivatives, greatly constrained target spot development.

The CHI and GLU are often linked with the resistance of plants to either abiotic and biotic stresses, and their importance against fungal pathogens is related to their capacity to hydrolyse chitin and β-1,3-glucan, respectively (Wu and Bradford 2003; Lattanzio et al. 2006; Gnanamangai et al. 2011). Accordingly, higher CHI and GLU activities were observed for the inoculated plants from cvs. TMG 32 and FUNDACEP 59 upon *C. cassiicola* infection. For the *Camellia sinensis*–*Cercospora theae* interaction, only CHI activity increased during fungal infection (Gnanamangai et al. 2011). In the present study, it was found that the CHI and GLU activities showed a similar pattern during the course of *C. cassiicola* infection, regardless of the soybean cultivar. In response to *Phytophthora infestans* infection, plants of *Solanum tuberosum* from susceptible and resistant cultivars exhibited a strong and coordinated increase in the activities of CHI and GLU (Schröder et al. 1992). Because the activities of CHI and GLU were similar in infected plants from cvs. TMG 132 and FUNDACEP 59, it seems plausible to assume that their higher activities were a general response of the soybean plants to constrain *C. cassiicola* infection.

In this study, the PAL activity was higher for the inoculated leaves relative to their non-inoculated counterparts regardless of the cultivar. The increase in PAL activity is of great importance for soybean resistance to target spot because it converts *L*-phenylalanine into *trans*-cinnamic acid, which in turn is the precursor of various phenylpropanoids such as lignin and flavonoids (Dixon et al. 2002; Hao et al. 2011; Borges et al. 2012). Although PAL activity increased during *C. cassiicola* infection on plants from cvs. TMG 132 and FUNDACEP 59, the difference between cultivars was negligible. In contrast with the results from the present study, the PAL activity in roots of pepper plants from a partially resistant cultivar was higher than for a susceptible cultivar after inoculation with *Phytophthora capsici* (Zhang et al. 2013). Iqbal et al. (2005) showed that during the infection of *F. solani*

Fig. 2 Activities of β-1-3-glucanases (GLU) (a and b), chitinases (CHI) (c and d), phenylalanine ammonia-lyases (PAL) (e and f), peroxidases (POX) (g and h), polyphenol oxidases (PPO) (i and j) and lipoxygenases (LOX) (k and l) in soybean leaves from cultivars TMG 132 (a, c, e, g, i and k) and FUNDACEP 59 (b, d, f, h, j and l) non-inoculated (NI) or inoculated (I) with *Corynespora cassiicola*. For each cultivar, the means between the NI and I treatments within each sampling time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. For either the NI or the I treatment, the means between cultivars within each sampling time that are followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard errors of the means. Two experiments were conducted with consistent results; the results of one representative experiment are shown.



(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Non-Inoculated
Inoculated
GLU (ΔAbs/min/mg protein)
CHI (nmol/min/mg protein)
PAL (μmol/min/mg protein)
POX (μmol/min/mg protein)
PPO (μmol/min/mg protein)
LOX (nmol/min/mg protein)
Days after inoculation
Days after inoculation

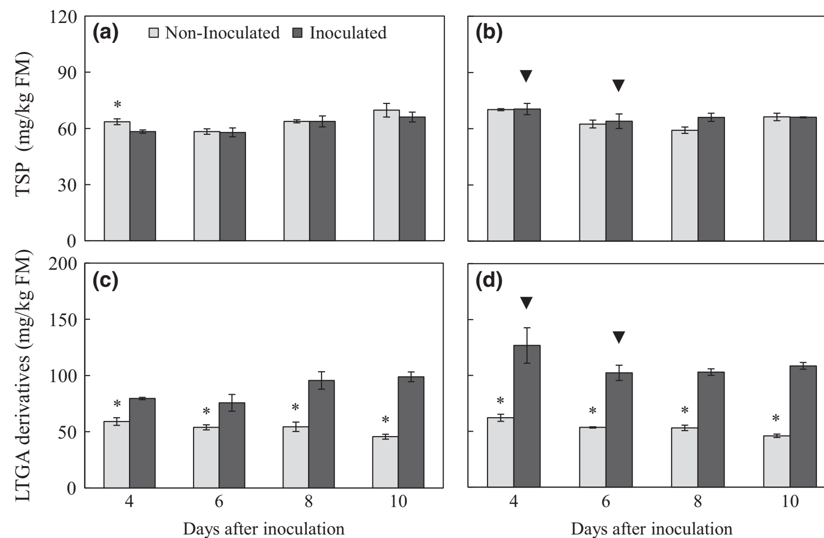


Fig. 3 Concentrations of total soluble phenolics (TSP) (a and b) and lignin-thioglycolic acid (LTGA) derivatives (c and d) in soybean leaves from cultivars TMG 132 (a and c) and FUNDACEP 59 (b and d) non-inoculated (NI) or inoculated (I) with *Corynespora cassiicola*. For each cultivar, the means between the NI and I treatments within each sampling time that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. For either the NI or the I treatment, means between cultivars within each sampling time that are followed by an inverted triangle (▼) are significantly different ($P \leq 0.05$) by *t*-test. The bars represent the standard errors of the means. Two experiments were conducted with consistent results; the results of one representative experiment are shown. FM = fresh matter.

f.sp. *glycines* on the roots of soybean plants from a resistant cultivar, the gene coding for the PAL enzyme was up-regulated, and this was not observed in the susceptible cultivar. Therefore, in the present study, PAL activity was also responsive to *C. cassiicola* infection, although the differences between cultivars were not large.

The increased phenolic levels provide an adequate substrate for oxidative reactions catalysed by PPO, which consume oxygen and produce fungitoxic quinones inside plant tissues reducing tissue colonization by pathogens (Lattanzio et al. 2006). In this study, PPO activity increased for plants from cvs. TMG 132 and FUNDACEP 59 in response to *C. cassiicola* infection. Accordingly, increases in the PPO activity were associated with increases in quinone levels, which, in turn, negatively affected the growth of *Phlyctaena vagabunda* (Lattanzio et al. 2001). Consistent with the results from the present study, Upchurch and Ramirez (2010) showed an up-regulation of the *PPO* gene in detached leaves of soybean plants and also in seeds infected by *C. kikuchii* or *D. phaseolorum* var. *meridionalis* compared to the non-infected leaf and seed tissues. In this study, the inoculated plants from cv. FUNDACEP 59 showed a more prominent increase in PPO activity than plants from cv. TMG 132, especially at the early stages of *C. cassiicola* infection. In agreement with these findings, Sahoo et al. (2009) reported an increase in the phenolics concentration and PPO activity on the leaves of resistant genotypes of taro compared to a susceptible genotype following *Phytophthora colocasia* infection. Therefore, in the present study, it is plausible that increases in PPO activity and in the concentration of LTGA derivatives played a pivotal role in soybean resistance to target spot.

The POX plays a key role in host defence responses to pathogen infections due to its participation in the production of hydrogen peroxide used for cell wall lignification or cross-linking with the cell wall proteins, in addition to its antimicrobial activity (Chittoor et al. 1999; Hiraga et al. 2001; Torres et al. 2006). Although the POX activity increased in response to *C. cassiicola* infection on plants from cvs. TMG 132 and FUNDACEP 59, differences between these cultivars were negligible. In contrast with these findings, the POX activity on the roots of pepper plants from resistant and partially resistant cultivars was more important than in the susceptible cultivar to counteract *P. capsici* infection (Zhang et al. 2013). Similarly, Leite et al. (2014) reported that POX activity was higher in plants of a resistant genotype of common bean in response to *Sclerotinia sclerotiorum* infection than for a susceptible genotype. In the present study, there was no difference between cultivars in POX activity, even considering its association with PPO in the polymerization of phenolic compounds, leading to an increase

Table 2 Pearson's correlation coefficients for target spot severity (TSS), the activities of β-1-3-glucanase (GLU), chitinase (CHI), phenylalanine ammonia-lyases (PAL), peroxidases (POX), polyphenol oxidases (PPO) and lipoxygenases (LOX) and the concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives in soybean leaves from cultivars TMG 132 and FUNDACEP 59 inoculated with *Corynespora cassiicola*

Variables	TSS^z	GLU	CHI	PAL	POX	PPO	LOX	TSP	LTGA
TSS	–	0.48^{ns}	0.14^{ns}	0.01^{ns}	0.61**	0.63**	−0.73**	0.47^{ns}	0.56*
GLU	0.11^{ns}	–	-0.08^{ns}	0.29^{ns}	0.27^{ns}	0.44^{ns}	-0.03^{ns}	0.12^{ns}	-0.09^{ns}
CHI	0.05^{ns}	0.04^{ns}	–	0.44^{ns}	0.17^{ns}	-0.29^{ns}	-0.30^{ns}	0.19^{ns}	0.12^{ns}
PAL	-0.04^{ns}	-0.23^{ns}	0.17^{ns}	–	-0.38^{ns}	-0.32^{ns}	-0.20^{ns}	0.28^{ns}	-0.18^{ns}
POX	0.65**	0.17^{ns}	0.28^{ns}	0.25^{ns}	–	0.43^{ns}	-0.28^{ns}	0.25^{ns}	0.27^{ns}
PPO	0.45^{ns}	0.56*	-0.17^{ns}	−0.53*	0.28^{ns}	–	-0.20^{ns}	0.13^{ns}	0.22^{ns}
LOX	−0.81**	0.02^{ns}	-0.26^{ns}	0.07^{ns}	−0.67**	-0.48^{ns}	–	-0.29^{ns}	−0.80**
TSP	-0.23^{ns}	-0.07^{ns}	0.30^{ns}	0.23^{ns}	-0.17^{ns}	-0.40^{ns}	0.35^{ns}	–	0.03^{ns}
LTGA	-0.05^{ns}	-0.19^{ns}	-0.04^{ns}	0.86**	0.15^{ns}	-0.41^{ns}	0.00^{ns}	-0.08^{ns}	–

^{z}Levels of probability: ns = not significant, * = 0.05 and ** = 0.01.

in tissue lignification around the infection site (Grisebach 1981; Vidhyasekaran 1988), thereby constraining the leaf tissue colonization by *C. cassiicola*.

The TSP can negatively affect pathogen growth by increasing membrane permeability and inhibiting the activity of secreted lytic enzymes (Tomás-Barberán et al. 1990; Lattanzio et al. 2006). In the present study, there were no differences between non-inoculated and inoculated plants, regardless of the cultivar, for the TSP concentration. For the bean–*Colletotrichum lindemuthianum* interaction, the reduction in the TSP concentration was associated with increases in the LTGA derivatives concentration (Polanco et al. 2012). In agreement with these results, it is believed that the TSP in soybean leaves infected by *C. cassiicola* may have been converted to LTGA derivatives. Additionally, the higher TSP concentration observed for the inoculated plants from cv. FUNDACEP 59 compared to plants from cv. TMG 132 could explain, at least in part, the increases in the LTGA derivatives concentration reported for the former cultivar. Similarly, a higher lignin concentration was reported to occur only in soybean roots from resistant lines in response to *F. solani* f.sp. *glycines* infection (Iqbal et al. 2005). It has been hypothesized that the deposition of lignin interferes with enzymatic hydrolysis and the mechanical penetration of plant tissue by fungal pathogens, thereby interfering with the movement of water and diffusible molecules such as non-selective toxins in the plant–fungus interface (Siranidou et al. 2002; Lattanzio et al. 2006). Thus, it seems plausible to hypothesize that a higher LTGA derivatives concentration contributed to the reduction of the deleterious effects of non-lytic enzymes and non-host selective toxins produced by *C. cassiicola* during its colonization of soybean leaf tissue, thereby contributing to soybean resistance to target spot.

The LOX catalyses the oxygenation of polyunsaturated fatty acids to produce hydroperoxides, which amplify specific host defence responses against pathogens or can irreversibly damage their plasma membrane (Hammond-Kosack and Jones 1996). In the present study, LOX activity in plants from cvs. TMG 132 and FUNDACEP 59 decreased in response to *C. cassiicola*. However, Hao et al. (2011) found an up-regulation of the *LOX* gene in both resistant and susceptible rice cultivars infected with the *rice stripe virus*. An increase in LOX activity was also reported to occur in rice leaves inoculated with either an incompatible and compatible race of *P. oryzae* (Ohta et al. 1991). Because LOX participates in the synthesis of jasmonic acid, a key hormone involved in plant defence against pathogen infections (Pieterse et al. 2009), it can be postulated that *C. cassiicola* was able to somehow modulate the LOX pathway for its own benefit, decreasing LOX activity.

In conclusion, the results of this study demonstrated that the defence-related enzyme activities increased upon *C. cassiicola* infection, regardless of the basal level of resistance of the cultivar studied. The increases in PPO activity and in the concentrations of TSP and LTGA derivatives, but lower LOX activity at early stages of *C. cassiicola* infection, were strongly associated with soybean resistance to target spot.

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