**ALESSANDRO ANTÔNIO FORTUNATO**

# BIOCHEMICAL CHANGES IN SOYBEAN LEAVES INFECTED BY *Corynespora cassiicola*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Doctor Scientiae*.

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APROVADA: em 31 de julho de 2014

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*Aos meus amados pais Maria A. Fortunato e*
*Antônio Fortunato, e amadas filhas*
*Maria Eduarda e Ana Luísa*

*OFEREÇO e DEDICO*

“***Live as if you were to die tomorrow.***

***Learn as if you were to live forever.”***

Mahatma Gandhi

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# BIOGRAFIA

ALESSANDRO ANTÔNIO FORTUNATO, filho de Maria Aparecida Fortunato e Antônio Fortunato, nasceu em 29 de setembro de 1982, em Nepomuceno, Estado de Minas Gerais. Ingressou em 2002, no curso de Agronomia da Universidade Federal Viçosa, Viçosa-MG, concluindo-o em março de 2007. Em agosto de 2007, iniciou o curso de Mestrado do Programa de Pós-Graduação em Fitopatologia na mesma instituição, concluindo-o em agosto de 2009. No período de setembro de 2009 a março de 2010, atuou como pesquisador associado no Departamento de Fitopatologia e Fisiologia de Culturas da Universidade Estadual da Louisiana, Baton Rouge-Louisiana-EUA. Em agosto de 2010, ingressou no curso de Doutorado do Programa de Pós-Graduação em Fitopatologia na Universidade Federal de Viçosa, Viçosa-MG, submetendo-se à defesa em 31 de julho de 2014.

# SUMÁRIO

# RESUMO

FORTUNATO, Alessandro Antônio, D. Sc., Universidade Federal de Viçosa, julho de 2014. **Alterações bioquímicas em folhas de soja infectadas por *Corynespora cassiicola***. Orientador: Fabrício Ávila Rodrigues. Coorientador: Luís Cláudio Viera da Cunha.

Amancha alvo da soja, causada pelo fungo *Corynespora cassiicola*, tornou-se uma importante doença em campos de soja no Centro-Oeste Brasil hoje em dia, causando perdas de produtividade de 20 a 40%. No entanto, informações sobre como as plantas de soja respondem à infecção de *C. cassiicola*, especialmente considerando o sistema de defesa antioxidantiva e a indução de defesa basal, ainda é incipiente. Portanto, o objetivo deste trabalho foi investigar as respostas bioquímicas de plantas de soja de duas cultivares (TMG 132 e FUNDACEP 59) à *C. cassiicola*. No primeiro estudo, foram medidas as atividades de superóxido dismutase (SOD), catalase (CAT), peroxidase (POX), peroxidase do ascorbato (APX), peroxidase da glutationa (GPX), glutationa redutase (GR), glutationa *S*-transferase (GST) e lipoxigenase (LOX), bem como as concentrações de ascorbato (ASA), aldeído malônico (MDA), peróxido de hidrogênio ($H_2O_2$) e de superóxido ($O_2^{\bullet-}$). A doença foi significativamente reduzida nos folíolos da cv. FUNDACEP 59 em comparação com a cv. TMG 132. Em geral, após a infecção as atividades de SOD, CAT, POX, APX, GPX, GR e GST foram significativamente maiores, independente da cultivar, em comparação com as plantas do controle. No entanto, a atividade de LOX em plantas de soja foi menor independente da cultivar. A concentração de AsA aumentou em ambas as cultivares em resposta a *C. cassiicola*. As cultivares apresentaram semelhanças quanto as respostas bioquímicas estudadas durante a patogênese. No entanto, as atividades de SOD, APX, GPX e GR em plantas inoculadas da cv. FUNDACEP e 59, bem como a concentração de AsA aumentaram em comparação com as mesmas plantas provenientes da cv. TMG 132. Foram observados aumentos significativos nas concentrações de MDA, $H_2O_2$ e $O_2^{\bullet-}$, especialmente nas plantas doentes da cv. TMG 132. Os resultados do presente estudo indicam que um sistema antioxidante mais eficiente em plantas de soja, especialmente para o cv. FUNDACEP 59, limitou os danos causados por ROS nos tecidos foliares. No segundo estudo, determinou-se as atividades de $\beta$-1-3-glucanase (GLU), quitinase (QUI), fenilalanina amônia-liase (FAL), peroxidase (POX), polifenol oxidase (PFO), bem como a concentração de compostos fenólicos solúveis totais (CFST) e lignina ácido-tioglicólico (LATG). Como observado no primeiro estudo, a doença foi significativamente reduzida na cv. FUNDACEP 59 em relação a cv. TMG 132. As atividades de GLU, QUI, FAL, POX e PFO, bem como a concentração de LATG aumentaram significativamente para as plantas inoculadas, independentemente da cultivar, em comparação com as não-inoculadas após a infecção fúngica. Além disso, diferenças significativas entre as plantas inoculadas de ambas as cultivares ocorreu apenas para PPO e LATG. Não houve diferença na concentração de TSP das plantas inoculadas, independentemente da cultivar, em comparação com as não-inoculadas durante a infecção. No entanto, após a infecção plantas da cv. FUNDACEP 59 tiveram um rápido e significativo aumento em TSP em comparação com as mesmas plantas provenientes da cv. TMG 132. Os resultados do presente estudo indicam que um aumento precoce na atividade da PPO e nas concentrações de TSP e LTGA em plantas de soja foram importantes para resistência à mancha alvo, especialmente para a cv. FUNDACEP 59.

# ABSTRACT

ALESSANDRO, Alessandro Antônio, D. Sc., Universidade Federal de Viçosa, July, 2014. **Biochemical changes in soybean leaves infected by *Corynespora cassiicola***. Adviser: Fabrício de Ávila Rodrigues. Co-adviser: Luís Cláudio Viera da Cunha.

Soybean target spot, caused by the fungus *Corynespora cassiicola*, has become an important disease on soybean fields in Midwest Brazil nowadays, causing yield losses from 20 to 40%. However, information regarding how soybean plants responds to *C. cassiicola* infection, especially considering the antioxidant defence system and the induction of basal defence, is still incipient. Therefore, the aim of this work was to investigate at the biochemical responses soybean plants from two cultivars (TMG 132 and FUNDACEP 59) to *C. cassiicola*. In the first study, it was measured the superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione *S*-transferase (GST) and lipoxigenase (LOX) activities as well as the concentrations of ascorbate (AsA), malondialdehyde (MDA), hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^{\bullet-}$). The disease was significantly reduced in leafleats from cv. FUNDACEP 59 compared to cv. TMG 132. In general, upon infection SOD, CAT, POX, APX, GPX, GR and GST activities were significantly higher, regardless of cultivar, in comparison to the control plants. However, LOX activity in soybean plants was lower upon infection regardless of cultivar. The concentration of AsA increased in both cultivars in response to *C. cassiicola*. The cultivars presented similarities regarding the biochemical responses studied during pathogenesis. However, SOD, APX, GPX and GR activities in inoculated plants from cv. FUNDACEP 59 and as well as the concentration of AsA were increased compared to the same plants from cv. TMG 132. There were observed significant increases in the concentrations of MDA, $H_2O_2$ and $O_2^{\bullet-}$, especially in diseased plants from cv. TMG 132. The results in the present study indicate that a more efficient antioxidant system in soybean plants, especially for cv. FUNDACEP 59, limited the damage caused by ROS in leave tissues. In the second study, it was determined the *ß*-1-3-glucanase (GLU), chitinase (CHI), phenylalanine ammonia-lyase (PAL), peroxidase (POX), polyphenoloxidase (PPO) activities as well as the concentration of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives. As observed in the first study, the disease was significantly reduced in the cv. FUNDACEP 59 compared to cv. TMG 132. GLU, CHI, PAL, POX and PPO activities as well as the concentration of LTGA derivatives significantly increased for the inoculated plants, regardless of cultivar, in comparison to the non-inoculated upon fungal infection. Moreover, significant differences between inoculated plants from both cultivars occurred only for PPO and LTGA derivatives. There was no difference in the concentration of TSP from inoculated plants, regardless of cultivar, compared to non-inoculated during infection. However, upon infection plants from cv. FUNDACEP 59 had an early and significant increase in TSP compared to the same plants from cv. TMG 132. The results from the present study indicate which an early increase in PPO activity and concentrations of TSP and LTGA in soybean plants were important for resistance to target spot, especially for the cv. FUNDACEP 59.

# GENERAL INTRODUCTION

(Sinclair, 1999). Brazil is the second the largest soybean (*Glycine max* (L.) Merril) producer in the world and largest exporter (FAO, 2014). In the 2013/14 growing season, the country produced about 89 million tons, accounting for 30% of the global production (CONAB, 2014).

Global soybean production for 2014/15 is projected at 304.8 million tons, up 4.8 million than last season (USDA, 2014). However, among the diseases affecting soybean production, soybean target spot, caused by the fungus *Corynespora cassiicola* (Berk & MA Curtis) CT Wei., has become important nowadays in fields due the losses caused in soybean production (Godoy *et al.*, 2012, Teramoto *et al.*, 2013). Among the reasons the over utilization of high yielding susceptible cultivars and continuous use of benzimidazole fungicides, resistant populations have been found in soybean fields in Goiás and Mato Grosso States (Xavier *et al.*, 2013).

The fungus *C. cassiicola* infects more than 372 host plants from over 70 tropical and subtropical countries (Farr & Rossman, 2014). Despite the wide range of host species, studies have shown that physiological specialization may occur in the field among isolates and their hosts (Dixon *et al.*, 2009). The fungus has been isolated from leaves, stems and roots of plants, and nematode cysts, human skin, and is able to produce chlamydospore (Dixon *et al.*, 2009, Huang *et al.* 2009; Oliveira *et al.*, 2012). Saprophytism and parasitism habits are common survival strategies for this pathogen, therefore endophytic isolates were recovered from rubber trees have been described recently in the literature (Déon *et al.* 2012).

Symptoms caused by *C. cassiicola* on soybean leaves include roughly circular to irregular necrotic lesions, which may have alternating light and dark rings surrounded by a dull green or yellowish-green halo (Almeida *et al.*, 2005; Sinclair, 1999). As the lesions expand, they start to coalesce and exhibit a zonate pattern contributing, therefore, to significantly decrease the photosynthetically active leaf area (Sinclair, 1999). Premature defoliation may occur in susceptible cultivars (Sinclair, 1999). The fungus overwinters on soybean debris and seed (Almeida *et al.*, 2005; Sinclair, 1999). Elevated temperatures associated with high relative humidity in mid to late season, especially when the plant canopy has been filled, are the environmental conditions that most favor the occurrence of severe epidemics of target spot (Godoy *et al.*, 2012; Teramoto *et al.*, 2012). However, due to the absence of cultivars with an adequate level of basal resistance to target spot and the low efficiency of chemical control by available registered fungicides, epidemics of target spot have been frequent in many soybean growing regions in Brazil (Godoy *et al.*, 2012; Teramoto *et al.*, 2013).

As an example of foliar and necrotrophic pathogenesis, *C. cassiicola* completely destroys the leaf cells as a strategy to obtain nutrients, thereby achieving massive growth, and the diffusion of secreted hydrolytic enzymes and non-host selective toxin (cassiicolin) into the healthy leaf tissues further increases the cellular damage (Lamotte *et al.*, 2006; Onesirosan *et al.*, 1975). The cellular damage caused by C. cassiicola results in the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen ion ($^{-}OH$), hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$) (Daub *et al.*, 2013). Because ROS are strong oxidizing agents, they can oxidase essential macromolecules such as membrane lipids, pigments, nucleic acids, carbohydrates, DNA and proteins, thereby leading to premature plant senescence (Gill & Tuteja, 2010). In order to maintain the balance between harmful and beneficial functions of ROS, their levels are strictly controlled by a flexible and complex antioxidant defence system orchestrated by the plants (Mandal *et al.*, 2008). In addition, ROS has been associated with plant defense due to their antimicrobial activity or contributing to the lignification of the host cell walls against pathogens colonization (Daub *et al.*, 2013; Grant & Loake, 2000).

During the necrotrophic phase of *B. cinerea* in tomato plants, the peroxisomal catalase activity on leaves was increased in the diseased plants at the first stages of fungal infection, however it was followed by a strong decline at the late stages compared to the healthy plants (Kużniak & Skłodowska, 2005). Differences between soybean cultivars for glutathione *S*-transferase activity expressing different resistance level to *Sclerotinia sclerotiorum* was reported by Malenčić *et al.* (2010). Soybean seedlings infected by *Rhizoctonia solani*, showed a significant increase for the superoxide dismutase activity on infected leaves in comparison to the healthy ones during the fungal infection process (Kiprovski *et al.*, 2012).

Plants also possess the ability to respond to infection through different mechanisms of defense, which include the expression of many defence-related genes, the synthesis of antimicrobial compounds such as phenolics and phytoalexins, production of ROS and tissue lignification (Hückelhoven, 2006; Lozovaya *et al.*, 2004; Upchurch & Ramirez, 2010). The great capacity of plants to reduce fungal colonization is likely to depend on the combination of these different defense mechanisms, which might vary according to the host-pathogen interaction (Hammond-Kosack & Parker, 2003).

For the soybean-*Fusarium solani* f.sp. *glycines* interaction, Lozovaya *et al.*, (2004) reported an increase in the concentration of lignin in the inoculated roots of soybean plants from resistant lines compared to the susceptible ones in response to fungal infection. Upchurch & Ramirez (2010) showed an up-regulation of polyphenol oxidase (PPO) gene on soybean detached leaves and seeds infected by *Cercospora kikuchii* or *Diaporthe phaseolorum* var. *meridionalis* compared to the control plants. The patterns of gene expression in soybean roots infected by *Phytophthora sojae* showed that the *PPO* gene was strongly down-regulated during the course of pathogen infection (Moy *et al.*, 2004). The genes coding for the enzymes phenylalanine ammonia-lyase and chalcone synthase and pathogenesis-related proteins PR1 and PR5 were up-regulated on the roots of plants from a resistant soybean cultivar to *F. solani* f.sp. *glycines* in comparison to a susceptible one (Iqbal *et al.*, 2005).

However, information regarding how soybean plants responds to *C. cassiicola* infection, especially considering the antioxidant defence system and the induction of basal defence, is lacking in the literature. Therefore, the aim of this work was to investigate at the biochemical responses in soybean plants from two cultivars (TMG 132 and FUNDACEP 59) to *C. cassiicola*.

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# *CHAPTER 1*

## Changes in the antioxidant system on soybean leaves infected by *Corynespora cassiicola*

Alessandro Antônio Fortunato, Daniel Debona, Arthur Martins Almeida Bernardeli and Fabrício Ávila Rodrigues

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## ABSTRACT

Target spot, caused by the fungus *Corynespora cassiicola*, has been very important foliar diseases of soybean nowadays. However, information regarding the soybean-*C. cassiicola* interaction is still scarse in the literature. In the present study, it was determined the superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST) and lipoxygenase (LOX) activities as well as the ascorbate (AsA), malondialdehyde (MDA), hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^{\bullet-}$) concentrations were studied in soybean plants from cultivars (TMG 132 and FUNDACEP 59). The target spot significantly decreased in in the leaflets of plants from cv. FUNDACEP 59 compared to cv. TMG 132. In general, the mentioned enzymes significantly increased their activities in the inoculated plants, regardless of cultivar, in comparison to the non-inoculated plants upon infection. Moreover, LOX activity interestingly was reduced in both cultivars. Upon pathogenesis, the enzyme activities were similar among both cultivars, however significant increases were observed to SOD, APX, GPX and GR activities. The concentration of AsA significantly increased upon *C. cassiicola* infection in both cultivars, especially for cv. FUNDACEP 59. The MDA, $H_2O_2$ and $O_2^{\bullet-}$ concentrations increased in response to *C. cassiicola* infection, especially for the plants from cv. TMG 132. Increases on SOD, POX, APX, GPX, GR and GST activities as well as on the AsA concentration in soybean plants were important to decrease the MDA concentration and the removal of the excess of ROS generated during to *C. cassiicola* infection.

Key words: antioxidant enzymes, cellular damage, target spot, *Glycine max*, oxidative stress.

# INTRODUCTION

Soybean (*Glycine max* (L.) Merrill), one of the most profitable crops grown worldwide, is susceptible to many diseases, which contribute to reduce grain quality and yield (Yorinori, 1997). In Brazil, target spot, caused by the necrotrophic fungus *Corynespora cassiicola* (Berk & MA Curtis) CT Wei., has increased in importance (Godoy *et al.*, 2012; Teramoto *et al.*, 2013). Symptoms the target spot on leaves include roughly circular to irregular necrotic lesions, which have alternating light and dark rings surrounded by a dull green or yellowish-green halo (Almeida *et al.*, 2005; Sinclair, 1999). As the lesions expand, they start to coalesce and exhibit a zonate pattern contributing, therefore, to significantly decrease the photosynthetically active leaf area (Sinclair, 1999). Premature defoliation may occur in susceptible cultivars (Almeida *et al.*, 2005; Sinclair, 1999). The fungus overwinters on soybean debris and seeds (Almeida *et al.*, 2005; Sinclair, 1999). Elevated temperatures associated with high relative humidity in mid to late season, especially when the plant canopy has been filled, are environmental conditions that favor the occurrence of severe epidemics of target spot (Godoy *et al.*, 2012). Target spot control has been difficult to achieve because of the absence of cultivars with a desirable level of resistance and the low efficiency and availability of fungicides (Xavier *et al.*, 2013, Teramoto *et al.*, 2013).

The secretion of lytic enzymes and non-host selective toxins, such as cassiicolin, by *C. cassiicola* (Barthe *et al.*, 2007; Onesirosan *et al.*, 1975) results in the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydroxyl ion ($^{-}OH$), hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$) (Daub *et al.*, 2013). ROS have been associated with plant defense due to their antimicrobial activity or even contributing to the lignification of the host cell walls that reduce the extension of pathogens colonization on their host tissue (Daub *et al.*, 2013; Grant & Loake, 2000). However, ROS are strong oxidizing agents that can damage essential macromolecules such as membrane lipids, pigments, nucleic acids, carbohydrates, DNA and proteins thereby leading to premature leaf senescence (Gill & Tuteja, 2010). To maintain an adequate balance between production and removal of the ROS, plants have a flexible and complex antioxidant system (Mittler *et al.*, 2004). This system involves a wide range of compounds including reduced ascorbate (AsA) and glutathione (GSH), carotenoids, flavonoids and other phenolics in addition to miscellaneous antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), lipoxygenase (LOX), ascorbate peroxidase (APX), glutathione reductase (GR), non-specific peroxidases (POX), glutathione peroxidase (GPX) and glutathione-*S*-transferase (GST) (Asada, 1999; Mittler, 2002, 2004; Malenčić *et al.*, 2010; Noctor & Foyer, 1998).

During the necrotrophic pathogenesis of *Botrytis cinerea* in tomato plants, the peroxisomal CAT activity on leaves was increased in the diseased plants at the first stages of fungal infection, however was followed by a strong decline at advanced stages compared to the healthy ones (Kużniak & Skłodowska, 2005). Increases in antioxidant system during fungal infection was associated with removal of ROS in soybean-*Sclerotinia sclerotiorum* interaction (Malenčić *et al.*, 2010). Soybean seedlings infected by *Rhizoctonia solani* showed a significant increase for SOD activity on infected leaves in comparison to the healthy ones (Kiprovski *et al.*, 2012).

In our study we hypothesized, that resistance to target spot could be associated with the potentiation of the anti-oxidative metabolism. Therefore, the main goal of the present study was to determine whether there is a marked difference in the efficiency of the antioxidant system between two soybean cultivars to *C. cassiicola* infection.

# MATERIAL AND METHODS

## Plant material and growth

A total of ten soybean seeds from cvs. TMG 132 and FUNDACEP 59, susceptible and moderately resistant to target spot based on field performance (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 an 1:1:1 mixture of pine bark, peat and expanded vermiculite. Five days after seedlings emergence, each pot was thinned to two seedlings. After the emergence of the cotyledons, plants were fertilized weekly with 50 mL of a nutrient solution, prepared using deionized water, 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 also were watered as needed with deionized water.The plants were kept in greenhouse (relative humidity of 65 ± 5% and temperature of 30 ± 5ºC) during the experiments.

## Inoculation procedure

A pathogenic isolate of *C. cassiicola* obtained from symptomatic soybean plants collected in the city of Rio Verde located in the State of Goiás, Brazil, was used to inoculate the plants. After isolation and morphological identification of conidia, the isolate was preserved by Castellani's method (Dhingra & Sinclair, 1995). At 14 days before inoculation, plugs of potato-dextrose-agar medium containing fungal mycelia preserved by Castellani’s method were placed in Petri dishes containing carrot leaf-pea-dextrose-agar (CL-PeDA) media. The CL-PeDA media was prepared using 200 mg carrot leaves, 100 g fresh pea, 20 g dextrose, and 20 g agar, and before media preparation, carrot leaves and peas were mixture in a blender to obtain a homogenous mixture, which was sieved to remove the solids excess. Fragments of fungal mycelia were transferred and homogenously spread to new Petri dishes. The Petri dishes were placed in a growth chamber at 25°C with a 12-h photoperiod for four days. After this period, the fungal colonies were stressed using a Drigalski spatel in a laminar flow chamber to avoid contamination. The plates were then kept in a growth chamber at continous white light (40 W lamps alternately distributed to provide the light intensity of 165.3 μmol $s^{-1}$ $m^{-2}$) for 6 days until the conidia were formed. Conidia were then carefully removed from the Petri dishes with a soft-bristle brush using water and 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* ($5 \times 10^4$ conidia $mL^{-1}$) (20 ml per plant). The conidial suspension was applied as a fine mist using a VL Airbrush atomizer (Paache Airbrush Co., Chicago, IL) to both adaxial and abaxial leaf surfaces of each plant until runoff. 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 plant canopy height was ≈ 700 $\mu mol\ m^{-2}\ s^{-1}$ and the temperature was 25 ± 2ºC (day) and 20 ± 2ºC (night). The relative humidity was maintained at 92 ± 3% using a misting system in which nozzles (model NEB-100, KGF Co., São Paulo, Brazil) sprayed mist every 30 min for 15 s above the plant canopy. The temperature and relative humidity were measured with a thermohygrograph (TH-508, Impac, Brazil).

## Disease assessment

Target spot severity was evaluated on the 8th trifoliate leaf from plants of each replication and treatment (cultivars) at 4, 6, 8, and 10 days after inoculation (dai) (based on personal greenhouse trials) using a diagrammatic scale proposed by Soares *et al.* (2009). Data from target spot severity was used to calculate the area under disease progress curve (AUDPC) according to Shaner & Finney (1977). At 10 dai, the number of lesions per $cm^2$ of leaf area was counted at five randomized places on on the 8th trifoliate leaves from the plants of each replication and cultivars with a hand-held microscope.

## Biochemical analysis

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 (cultivars × plant inoculation) were collected at 4, 6, 8 and 10 dai (based on personal greenhouse trials). Leaf samples were kept in liquid nitrogen during samplings and subsequently stored at -80°C until further analysis.

## Determination of enzymes activities

To determine the superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.7), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione peroxidase (GPX, EC 1.11.1.9) and glutathione-S-transferase (GST, EC 2.5.1.18)activities, 200 mg of homogenized leaf tissue was ground into a fine powder in a mortar using a pestle adding liquid nitrogen. The fine powder was immediately homogenized in 2000 µL of a solution containing 50 mM potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% (w/v) polyvinylpolypyrrolidone (PVPP). Then, the homogenate was centrifuged at 12,000 *g* for 15 min at 4°C and the supernatant was used as a crude enzyme extract. To determine the glutathione reductase (GR, EC 1.6.4.2) activity, 200 mg of homogenized leaf tissue was ground as described above and the extraction solution used to homogenize the fine powder containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 1 mM *DL*-dithiothreitol, 1 mM PMSF and 2% (w/v) PVPP in final volume of 2000 µL. The homogenate was centrifuged as described previously.

SOD activity was determined using a method described by Del Longo *et al.* (1993), which measures its capacity to photochemically reduce the *p*-nitrotetrazolium blue (NTB) available in the reaction solution. The reaction was started after the addition of 40 µL of the crude enzyme extract to 960 µL of a mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NTB, 0.1 mM EDTA and 2 µM riboflavin. The reaction was carried out at 25°C under 15-W lamp light per 10 min. After light exposure, the light was turned off and the production of formazan blue, which resulted from the photoreduction of NTB, was measured at 560 nm in spectrophotometer (Evolution 60; Thermo Fisher Scientific Inc., MA) (Giannopolitis & Ries, 1977). For the control samples, the reaction mixture was kept in darkness for 10 min and the absorbance measured at 560 nm. The values obtained from the samples (light) were subtracted from the values obtained from the control samples to determine the SOD activity. The amount of enzyme necessary to inhibit NBT photoreduction by 50% was defined as one unit of SOD (Beauchamp & Fridovich, 1971).

CAT activity was determined following the method proposed by Cakmak & Marschner (1992). The reaction was initiated after the addition of 25 µL of the crude enzyme extract to 975 µL of reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.8) and 20 mM $H_2O_2$. The determination of CAT activity was based by the rate of hydrogen peroxide ($H_2O_2$) decomposition measured in the spectrophotometer at 240 nm for 1 min at 25°C. An extinction coefficient of 36 $M^{-1}$ $cm^{-1}$ was used to calculate CAT activity (Anderson *et al.*, 1995).

POX activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar & Mishra (1976). The reaction was started after the addition of 15 µL of the crude enzyme extract to 985 µL of reaction mixture containing 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol and 20 mM $H_2O_2$. The POX activity was obtained through the absorbance of colored purpurogallin recorded at 420 nm for 1 min at 25°C. The extinction coefficient of 2.47 $mM^{-1}$ $cm^{-1}$ was used to calculate POX activity (Chance & Maehley, 1955).

APX activity was assayed using the method proposed by Nakano & Asada (1981). The reaction was started after the addition of 25 µL of the crude enzyme extract to 975 µL of reaction mixture consisted by 50 mM potassium phosphate buffer (pH 6.8), 1 mM $H_2O_2$ and 0.8 mM ascorbate. The APX activity was measured by the rate of ascorbate oxidation at 290 nm for 1 min at 25°C. The extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$ was used to calculate APX activity (Nakano & Asada, 1981).

GPX activity was determined after the addition of 50 µL of the crude enzyme extract to a 950 µL of mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.114 M NaCl, 1 mM GSH, 0.2 mM NADPH, 0.25 mM $H_2O_2$ and 1 U of GR (Nagalakshmi & Prasad, 2001). The enzyme activity was measured as a decrease in absorbance at 340 nm for 1 min at 30°C. The extinction coefficient of 6.22 $mM^{-1}$ $cm^{-1}$ was used to calculate GPX activity (Anderson *et al.*, 2004).

GR activity was assayed according to method described by Carlberg & Mannervik (1985). The reaction was started after the addition of 50 µL of the crude enzyme extract to 950 µL of a mixture containing 100 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1 mM oxidized glutathione (GSSG) and 0.1 mM NADPH prepared in 0.5 mM Tris-HCl buffer (pH 7.5). The decrease in absorbance was determined at 340 nm for 1 min at 30°C. The extinction coefficient of 6.22 $mM^{-1}$ $cm^{-1}$ was used to calculate GR activity (Foyer & Halliwell, 1976).

The methodology proposed by Habig *et al.* (1974) was used to determined the GST activity. Firstly, 75 µL of the crude enzyme extract was add to 925 µL of a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5) and 50 mM reduced glutathione (GSH). Then, the reaction was started by the addition of 500 µL of 30 mM 1-chloro-2,4-dinitrobenzene and the absorbance was measured at 340 nm over 3 min at 25°C. GST activity was determined using an extinction coefficient of 9.6 $mM^{-1}$ $cm^{-1}$ (Habig *et al.*, 1974).

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 a volume of 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 LOX for 1 min was measured in a spectrophotometer at 234 nm. The extinction coefficient of 25,000 $M^{-1}$ $min^{-1}$ was used to calculate LOX activity.

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

### Determination of reduced ascorbate concentration

The concentration of reduced ascorbate (AsA) was determinated following the method described by Kampfenkel *et al.* (1995). A total of 300 mg of leaf tissue was homogenized in 2000 µL of 6% (w/v) acid trichloroacetic (TCA) and centrifuged at 15,000 *g* for 5 min at 4°C. Aliquots of 200 µL of the crude extract leaf were added to 800 µL of 0.2 M sodium phosphate buffer (pH 7.4). The mixture was incubated at 42°C for 15 min. Subsequently, 1.0 mL of 10% (w/v) TCA, 800 µL of 42% (v/v) $H_3PO_4$, 800 µL of 4% (w/v) 2,2'-dipyridyl (dissolved in 70% ethanol) and 400 µL of 3% (w/v) $FeCl_3$ were added to the mixture. After vigorous stirring, the mixture was incubated at 42°C for 40 min. Then, the reaction was stopped on ice bath and the absorbance was determined at 525 nm. The AsA concentration was determined according to a calibration curve of AsA.

### Determination of malondialdehyde (MDA) concentration

The oxidative damage in leaf cells was determined as the concentration of total 2-thiobarbituric acid (TBA) reactive substances and expressed as equivalents of malondialdehyde (MDA) according to Cakmak & Horst (1991). Firstly, 100 mg of leaf tissue was ground into a fine powder using a mortar with pestle adding liquid nitrogen. Then, the fine powder was homogenized in 2000 µl of 0.1% (w/vol) trichloracetic acid (TCA) solution. The homogenate was centrifuged at 12,000 *g* for 15 min at 4°C. After centrifugation, 500 µL of the supernatant was reacted with 1500 µL of TBA solution (0.5% in 20% TCA) in ThermoMixer at 95°C for 30 min (Eppendorf, Hamburg, Germany). After this period, the reaction was stopped in an ice bath. The samples were centrifuged at 9,000 *g* for 10 min and the specific absorbance was determined at 532 nm. The nonspecific absorbance was estimated at 600 nm and subtracted from the specific absorbance value. An extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$ was used to calculate the MDA concentration (Heath & Packer, 1968).

### Determination of hydrogen peroxide ($H_2O_2$) concentration

The method described by Kuo & Kao (2003) was used to assay the $H_2O_2$ concentration. Initially, 100 mg of homogenized leaf tissue was ground into a fine powder using a mortar with pestle adding liquid nitrogen. The fine powder was homogenized with a extraction mixture containing 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine in a volume of 2000 µL. The homogenate was centrifuged at 10,000 *g* for 15 min at 4°C and the supernant collected. The reaction was initiated with addition of 100 µL of the supernatant to the reaction mixture containing 100 µM ferric ammonium sulfate ($FeNH_4[SO_4]$), 25 mM sulfuric acid, 250 µM xylenol orange and 100 mM sorbitol in a volume of 2000 µL (Gay & Gerbicki, 2000). The samples were kept in the darkness for 30 min and then the absorbance was determined at 560 nm. The controls for the reagents and crude extracts were prepared under the same conditions and subtracted from the sample. The $H_2O_2$ concentration was estimated based on a standard curve of $H_2O_2$.

## Determination of superoxide ($O_2^{\bullet -}$) concentration

A total of 200 mg of leaf tissue was ground into a fine powder in a mortar and pestle with liquid nitrogen to determine the $O_2^{\bullet -}$ concentration. The fine powder was homogenized in an ice bath in 2000 µL of a solution containing 100 mM sodium phosphate buffer (pH 7.2) and 1 mM sodium diethyl diethyldithiocarbamate. The homogenate was centrifuged at 22,000 *g* for 20 min at 4°C. After centrifugation, 100 µL of the supernatant was reacted with 1900 µL of a solution containing 100 mM sodium phosphate buffer (pH 7.2), 1 mM diethyl sodium diethyldithiocarbamate and 0.25 mM *p*-nitrotetrazolium blue. The $O_2^{\bullet -}$ concentration was determined by subtracting the absorbance of the final product from the initial absorbance at 540 nm (Chaitanya & Naithani, 1994).

## Experimental design and data analysis

An experiment with 12 replications consisting of two cultivars (TMG 132 and FUNDACEP 59) inoculated with *C. cassiicola* was arranged in a completely randomized design to evaluate the target spot severity, NLL and AUDPC. Another 2 × 2 × 4 factorial experiment with four replications consisting of two cultivars, non-inoculated or inoculated plants and four sampling times with a completely randomized design was used to obtain the samples for the biochemical analysis. For severity, the ANOVA was considered a 2 × 4 factorial experiment consisting of two cultivars and four evaluation times (4, 6, 8 and 10 dai). For NLL and AUDPC, the ANOVA was considered a one-way ANOVA consisting of two cultivars. For the biochemical variables, the ANOVA was considered a 2 × 2 × 4 factorial consisting of two cultivars, plant inoculation (non-inoculated and inoculated plants) and four sampling times (4, 6, 8 and 10 dai). Each experimental unit was consisted of a 2 L plastic pot with two plants. Data from all variables were analyzed by analysis of variance (ANOVA) and means from the treatments were compared using *t*-tests ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). The Pearson correlation technique was used to determine the relationships among severity and the SOD, CAT, POX, APX, GPX, GST and LOX activities as well as the concentrations of AsA, MDA, $H_2O_2$ and $O_2^{\bullet -}$.

## RESULTS

The factors cultivars and sampling time as well as their interaction were significant for target spot severity (Table 1). For the NLL and AUDPC, the factor cultivars was significant (Table 1). The symptoms of target spot, characterized as circular brownish lesions with yellow halos, were more developed on the leaflets of plants from cv. TMG 132 in comparison to cv. FUNDACEP 59 (Fig. 1A). The NLL was significantly reduced by 14% for plants from cv. FUNDACEP 59 compared to cv. TMG 132(Fig. 1B). The target spot severity was significantly reduced by 50, 13, 21 and 19% at 4, 6, 8 and 10 dai, respectively, on the leaflets of plants from cv. FUNDACEP 59 in comparison to the leaflets of plants from cv. TMG 132 (Fig. 1C). The AUDPC was significantly lower by 15% on the leaflets of plants from cv. FUNDACEP 59 in comparison to the leaflets of plants from cv. TMG 132 (Fig. 1D).

The factors cultivars, plant inoculation and sampling time were significant for SOD, POX, APX, GR and LOX activities as well as for the AsA and $O_2^{\bullet-}$ concentrations (Table 1). For CAT and GST activities, only the factors plant inoculation and sampling time were significant. Only the factors cultivars and plant inoculation were significant for GPX activity and the MDA and $H_2O_2$ concentrations. The SOD, CAT, POX, APX, GPX, GR, GST and LOX activities as well as the AsA, MDA, $H_2O_2$ and $O_2^{\bullet-}$ concentrations were significantly influenced by at least one of the 2-way and 3-way interactions (Table 1). For cvs. TMG 32 and FUNDACEP 59, POX, GPX and GST activities were significantly increased upon fungal inoculation (Fig. 2). For cv. TMG 32, inoculated plants had significant higher activities of SOD at 6, 8 and 10 dai, CAT at 6 dai, APX at 4 dai and GR at 4 and 10 dai in comparison to the non-inoculated plants (Fig. 2). For cv. FUNDACEP 59, the inoculation with *C. cassiicola* resulted in a significant increase on the activities of SOD at 6, 8 and 10 dai, CAT at 6 and 10 dai, APX at 4, 8 and 10 dai and GR at 4, 6 , 8 and 10 dai (Fig. 2). For the non-inoculated plants, significant difference between the cvs. TMG-32 and FUNDACEP 59 occurred only for GST at 4 dai, with higher values recorded for the former cultivar (Fig. 2). For the inoculated plants from cv. FUNDACEP 59, higher activities were observed for SOD at 8 and 10 dai, APX at 4 and 10 dai, GPX at 4 and 6 dai, GR at 6 and 10 dai and GST at 4 dai, whereas a lower POX activity occurred at 4 dai in comparison to the inoculated plants from cv. TMG 132 (Fig. 2). In general, for cv. TMG 32, the concentrations of AsA, MDA, $H_2O_2$ and $O_2^{\bullet-}$ were significantly higher for the inoculated plants upon infection, respectively, in comparison to the non-inoculated counterparts (Fig. 3). However, for cv. FUNDACEP 59, only AsA and $O_2^{\bullet-}$ increased after infection by *C.*

*cassiicola*, compared with the non-inoculated ones. The LOX activity significantly decreased for the inoculated plants in comparison to the non-inoculated counterparts during pathogenesis (Fig. 3). Higher AsA concentration was found upon infection for the inoculated plants from cv. FUNDACEP 59 in contrast to plants from cv. TMG 132. Moreover, inoculated plants from cv. FUNDACEP 59 showed lower values for the MDA $H_2O_2$ and $O_2^{\bullet-}$ concentrations in response to *C. cassiicola* in comparison to the inoculated plants from cv. TMG 132 (Fig. 3).

For cv. TMG 132, there was a positive correlation of target spot severity with POX, GPX, and the $O_2^{\bullet-}$ concentrations, whereas a negative correlation occurred with LOX (Table 2). SOD correlated positively with POX and with the AsA concentration, but was negatively correlated with GST. CAT correlated negatively with GR and POX positively correlated with APX. POX positively correlated with AsA. GST and LOX were negatively correlated with the $O_2^{\bullet-}$concentration (Table 2). For the cv. FUDANCEP 59, there was a positive correlation of target spot severity with SOD, POX, APX and the $O_2^{\bullet-}$ concentration, but negatively correlated with LOX (Table 2). There was a positive correlation of POX with APX and the $O_2^{\bullet-}$ concentration, whereas CAT correlated negatively with GST (Table 2). LOX correlated positively with the $O_2^{\bullet-}$ concentration, but correlated negatively with POX (Table 2).

## DISCUSSION

Plant cells exposed to pathogens produce ROS such as $O_2^{\bullet-}$, $^{-}OH$, $H_2O_2$ and $^1O_2$ in response to their infection (Daub *et al.*, 2013; Knörzer *et al.*, 1999). The imbalance between the ROS produced by the pathogens and by their hosts cause an accumulation of ROS that results in damage to the host cells that promotes pathogen colonization (Daub *et al.*, 2013; Heller & Tudzynski, 2011). However, the ROS also has been associated with plant defense against pathogens due to their antimicrobial activity or contributing to the strengthening of host cell walls via lignification resulting in reduce disease symptoms on leaves (Daub *et al.*, 2013; Grant & Loake, 2000). The present study brings novel evidence that a more efficient antioxidative system on the leaves of soybean plants is essential to reduce the damages caused ROS associated with the infection caused by *C. cassiicola*. The reduced target spot severity, NLL and AUDPC in the leaves of plants from cv. FUNDACEP 59 compared to plants from cv. TMG 132 confirmed the variation in their resistance as previously reported by Godoy *et al.* (2012) and Pitol *et al.* (2011). However, information regarding the possible mechanisms involved in soybean resistance to target spot is lacking, in the literature. A more efficient antioxidant system in the leaves of plants from cv. FUNDACEP 59 could explain their higher resistance to target spot in removing the excess of ROS and, consequently, reducing the cellular damage caused by *C. cassiicola* infection.

Lipid peroxidation is considered as the most damaging process known to occur in plant cells and membrane damage is sometimes taken as a single physiological variable to determine the level of lipid destruction under various stresses especially by ROS (Apel & Hirt, 2004; Blokhina & Fagerstedt, 2010). The ROS may induce the peroxidation of unsaturated fatty acids in the membranes leading to the formation of lipid peroxidation products such as MDA (Mishra *et al.*, 2008). The high ROS concentration in the roots of tomato plants infected by *F. oxysporum* f.sp. *lycopersici* resulted in increased lipid peroxidation (Mandal *et al.*, 2008). In the present study, the $O_2^{\bullet-}$, $H_2O_2$ and MDA concentrations for inoculated plants from cv. TMG 132 were higher than for the non-inoculated counterparts. The lower activities of scavenger enzymes on inoculated plants could explain the high level of ROS and MDA concentrations. On the other hand, for plants from cv. FUNDACEP 59 there was only an increase in $O_2^{\bullet-}$ in the late stage of fungal infection in comparison to the non-inoculated plants. Debona *et al.* (2012) showed that wheat plants from a susceptible cultivar showed a significant increase in the MDA, $O_2^{\bullet-}$ and $H_2O_2$ concentrations in comparison to plants from a resistant cultivar during infection by *P. oryzae*. In the present study, increases in the MDA, $O_2^{\bullet-}$ and $H_2O_2$ concentrations were observed only for inoculated plants from cv. TMG 132. The lower target spot severity on plants from cv. FUNDACEP 59 than for plants from cv. TMG 132 could be associated with the increases in the scavengers enzymes examined in the present study and with high AsA concentration and the interaction of these ROS scavengers could avoid the accumulation of ROS and the MDA formation.

SOD is one of the most important scavenging enzymes and represents the first line of defence catalyzing the dismutation of $O_2^{\bullet-}$ to $H_2O_2$ and oxygen ($O_2$) (Gill & Tuteja, 2010; Giannopolitis & Ries, 1977; Hao *et al.*, 2011). SOD activity increased on the leaves of plants from both cultivars in response to *C. cassiicola* infection; however, we hypothesized that a more prominent increase in SOD activity for plants from the cv. FUNDACEP 59 showed in the early stage of fungal infection, could contributed to reduce $O_2^{\bullet-}$ concentration. Kiprovski *et al.* (2012), studying the antioxidant responses of soybean seedlings in response to *Rhizoctonia solani* infection, showed that SOD activity on infected leaves was lower than in the leaves of non-inoculated plants resulting in a higher $O_2^{\bullet-}$ concentration in their tissue. In tomato, the peroxisomal SOD activity increased at the initial stages of *Botrytis cinerea* infection, but decreased as the necrotic lesions appeared (Kużniak & Sklodowska, 2005). SOD activity increased in maize ears infected by *Fusarium verticillioides* for the resistant cv. CO44 compared to the susceptible cv. CO354 (Lanubile *et al.*, 2012). The fungus *C. cassiicola* could exploit the increase in ROS species, especially the $O_2^{\bullet-}$, generated during its interaction with the soybean leaf cells for its own benefit. However, a higher SOD activity on the leaves of plants from cv. FUNDACEP 59 could be one of the strategies to restrict fungal colonization.

CAT is responsible for the detoxification of the excess $H_2O_2$ generated spontaneously or by $O_2^{\bullet-}$ dismutation via SOD (Lanubille *et al.*, 2012). Higher CAT activity occurred only in the leaves of inoculated plants from cv. FUNDACEP 59 at 6 and 10 dai in comparison to the non-inoculated plants. Debona *et al.* (2012) showed that an increase in the concentration of $H_2O_2$ in the leaves of the *Pyricularia oryzae* inoculated plants of a susceptible cultivar compared with the non-inoculated plants was associated with a reduction in CAT activity. It was detected that a decrease on CAT activity on the leaves of inoculated plants compared to non-inoculated counterparts could be associated with the increases in $H_2O_2$ concentration. The peroxisomal CAT activity on tomato leaves in response to *B. cinerea* infection was higher in the diseased plants at the first stages of fungal infection, but it was followed by a strong decline at the late stages compared to the non-inoculated plants (Kużniak & Sklodowska, 2005). Lanubile *et al.* (2012) demonstrated that for the maize-*F. verticillioides* interaction, no significant changes occurred for CAT activity on inoculated ears from a resistant cultivar. Nevertheless, in the present study, CAT activity, associated with the other antioxidant enzymes, could efficiently maintain the $H_2O_2$ concentration at the same level as detected for the non-inoculated plants from cv. FUNDACEP 59.

POX plays an important role in the host defence through the process of antioxidant protection by catalyzing the oxidation of $H_2O_2$ (Mittler *et al.*, 2004; Gill & Tuteja, 2010). POX also participates in the synthesis of lignin, which strengthens the cell wall against the action of lytic enzymes produced in abundance by several pathogens (Chittoor *et al.*, 1999). In the present study, there was a consistent increase in POX activity for inoculated plants of both cultivars in comparison to the non-inoculated counterparts; nevertheless, the increase was more pronounced for plants from cv. FUNDACEP 59 at early stage of fungal infection than for the susceptible cultivar. In agreement with the data from the present study, Hong-xia *et al.* (2011) showed that for the wheat-*Rhizoctonia cerealis* interaction, POX activity was higher for a resistant than for a susceptible wheat cultivar in response to fungal infection. Great POX activity was maintained at a higher level in the leaves of wheat plants of resistant cultivar than in the susceptible during the infection process of *P. oryzae* (Debona *et al.*, 2012). Based on the roles of POX as ROS scavenger and lignification process, the higher POX activity in the inoculated plants, especially for the cv. FUNDACEP 59 could somehow act directly catalyzing the oxidation of $H_2O_2$ or participating in the lignin biosynthesis.

APX is responsible by the removal of $H_2O_2$ in the plant tissue using AsA, considered as a most powerful ROS scavenger, as electron donor to reduce $H_2O_2$ to water (Foyer & Noctor, 2011). In the present study, APX activity on inoculated plants from cv. TMG 132 was higher than for the non-inoculated counterparts at early stage of fungal infection and the AsA concentration in the same plants at advanced stages of fungal infection. El-Zahaby *et al.* (1995) demonstrated an increase in APX activity in the leaves of susceptible barley cultivars than in the resistant ones during infection with *Erysiphe graminis* f.sp. *hordei*. In the present study, APX activity and the concentration of AsA were greater for the inoculated plants in comparison to the non-inoculated plants from cv. FUNDACEP 59. A higher APX activity was observed on the leaves of plants from a wheat resistant cultivar to blast than on the susceptible cultivar (Debona *et al.*, 2012). Polkowska-Kowalczyk *et al.* (2007) demonstrated that for the *Solanum tuberosum-Phytophthora infestans* interaction, APX activity as well as the concentration of AsA were significantly higher for the resistant cultivar than for the susceptible ones. These authors suggest that APX activity and AsA played an important role in the regulation of ROS levels upon *P. infestans* infection. Indeed, the balance between SOD activity and the $H_2O_2$-scavenging enzymes, such as APX, is considered to be crucial in determining the steady-state level of $H_2O_2$ and $O_2^{\bullet-}$ in plant cells (Lanubille *et al.*, 2012). Thus, the increased SOD and APX activities and the high AsA concentration for the inoculated plants from cv. FUNDACEP 59 could prevent the excess of $O_2^{\bullet-}$ and the toxic effect of $H_2O_2$ over-accumulation.

GPX and GST are important enzymes on pathogen-infected plants and use available GSH to reduce $H_2O_2$ and other hydroperoxides alleviating the oxidative stress in plant cells (Gill & Tuteja, 2010). For the inoculated plants from cvs. TMG 132 and FUNDACEP 59, there was a consistent increase in the GPX and GST activities during *C. cassiicola* infectious process compared to the non-inoculated plants. However, Debona *et al.* (2012) observed increases in the GPX activity only for the inoculated plants from the susceptible wheat cultivar to blast compared to the non-inoculated ones. In the present study, differences between cultivars regarding GPX activity occurred only at the early stages of fungal infection. GST activity was more pronounced for plants of cv. FUNDACEP 59 especially at 4 dai than for plants of cv. TMG 132. Polkowska-Kowalczyk *et al.* (2007) demonstrated differences in GST activity between susceptible and resistant genotypes of *S. tuberosum* inoculated with *P. infestans* in which the resistant genotype showed lower GST activity than the susceptible one. Therefore, in the present study, plants from cv. FUNDACEP 59 showed a more pronounced increase on GST activity during *C. cassiicola* infectious process. GPX and GST activities for plants from cv. FUNDACEP 59 were slightly higher than for plants from cv. TMG 132, which could explain the reduced disease severity as well as associated with a more efficient ROS removal in the plant tissue of plants from the former cultivar.

GR belongs to the Foyer-Halliwell-Asada pathway, whose function is to scavenging ROS in the plastids and possibly in extra-plastidic compartments of the plant cell (Foyer & Noctor, 2011; Noctor & Foyer, 1998). GR also maintain the reducing power of this cycle by regenerating the two major ROS scavengers in plant cells, ascorbate and glutathione (Foyer & Noctor, 2011). GR activity was higher for the inoculated plants than for the non-inoculated ones, especially for cv. FUNDANCEP 59. Conversely, at the early stage of *B. cinerea* infection on tomato leaves, GR activity decreased in comparison to the non-infected tissue (Kużniak & Sklodowska, 2005). In the apricot-*Plum pox virus* interaction, GR activity in the leaves of plants from a resistant cultivar was higher than in the susceptible one following virus infection (Hernández *et al.*, 2001). In a study involving the interaction between *S. tuberosum* and an elicitor derived from *P. infestans*, the increase on GR activity at an early phase of culture filtrate treatment was higher in the susceptible than in the resistant cultivars (Polkowska-Kowalczyk *et al.*, 2007). However, data from the present study suggest that a higher increase in GR activity in the plants from cv. FUNDANCEP 59 than for cv. TMG 132 at the first and late stages of fungal infection could be important to the removal of excess of $H_2O_2$.

The LOX catalyzes the oxygenation of poly-unsaturated fatty acids to produce hydroperoxides, which are metabolized to compounds such as jasmonic acid, methyl jasmonate or lipid peroxides, which coordinately amplify specific defence responses against pathogens infection (Hao *et al.*, 2011). Moreover, LOX activity may also cause irreversible membrane damage, which would lead to the leakage of cellular contents resulting in the production of toxic volatile and non-volatile fatty acid-derived secondary metabolites that could directly attack invading pathogens (Hammond-Kosack & Jones, 1996). An increase in LOX activity in response to pathogens infection has been reported for many host-pathogen interactions (Slusarenko, 1996). Hao *et al.* (2011) found an induced expression of *LOX* gene in both resistant and susceptible rice cultivars infected with the *Rice stripe virus*. In contrast, data from the present study showed a different response regarding LOX activity upon plants from cvs. TMG 132 and FUNDACEP 59 were inoculated with *C. cassiicola*. The LOX activity for the non-inoculated plants of both cultivars was higher than for the inoculated counterparts. Increased LOX activity occurred on rice leaves inoculated with an incompatible race of *P. oryzae* rather than for a compatible race (Ohta *et al.*, 1991). LOX activity is associated with mechanisms that act direct or indirectly in response to pathogens attack (Hammond-Kosack & Jones, 1996; Hao *et al.*, 2011). However, we hypothesized that the *C. cassiicola* somehow is interfering in the expression of *LOX* gene that culminate in a reduced LOX activity on the leaves of both cultivars upon *C. cassiicola* infection in comparison to the non-inoculated plants. However, as LOX is also important as peroxidation enzyme, the low levels of MDA in cv. FUNDACEP 59 could be associated to the less damage in the cells of this cultivar compared to the cultivar more susceptible.

The results from the present study indicate that a more efficient antioxidative system in the removal of the excess of ROS generated during *C. cassiicola* pathogenesis in soybean leaves limited, at a certain level, the cellular damage caused by this fungus contributing, therefore, to a greater resistance to target spot. Further research aiming to investigate the antioxidative system in the most productive soybean cultivars will play a pivotal role in the development of biochemical markers that can be used in breeding programs to select cultivars that can be grown in areas in which severe epidemics of target spot often occur.

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**Table 1** Analysis of variance of the effects of cultivars (C), plant inoculation (PI) and sampling time (ST) on the target spot severity (SEV), number of lesions per leaflet (NLL), area under disease progress curve (AUDPC), and the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST) and lipoxigenase (LOX) as well as on the concentrations of ascorbate (AsA), malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^{\bullet -}$) in the leaves of soybean plants from cvs. TMG 132 and FUNDACEP 59 inoculated with *Corynespora cassiicola*.

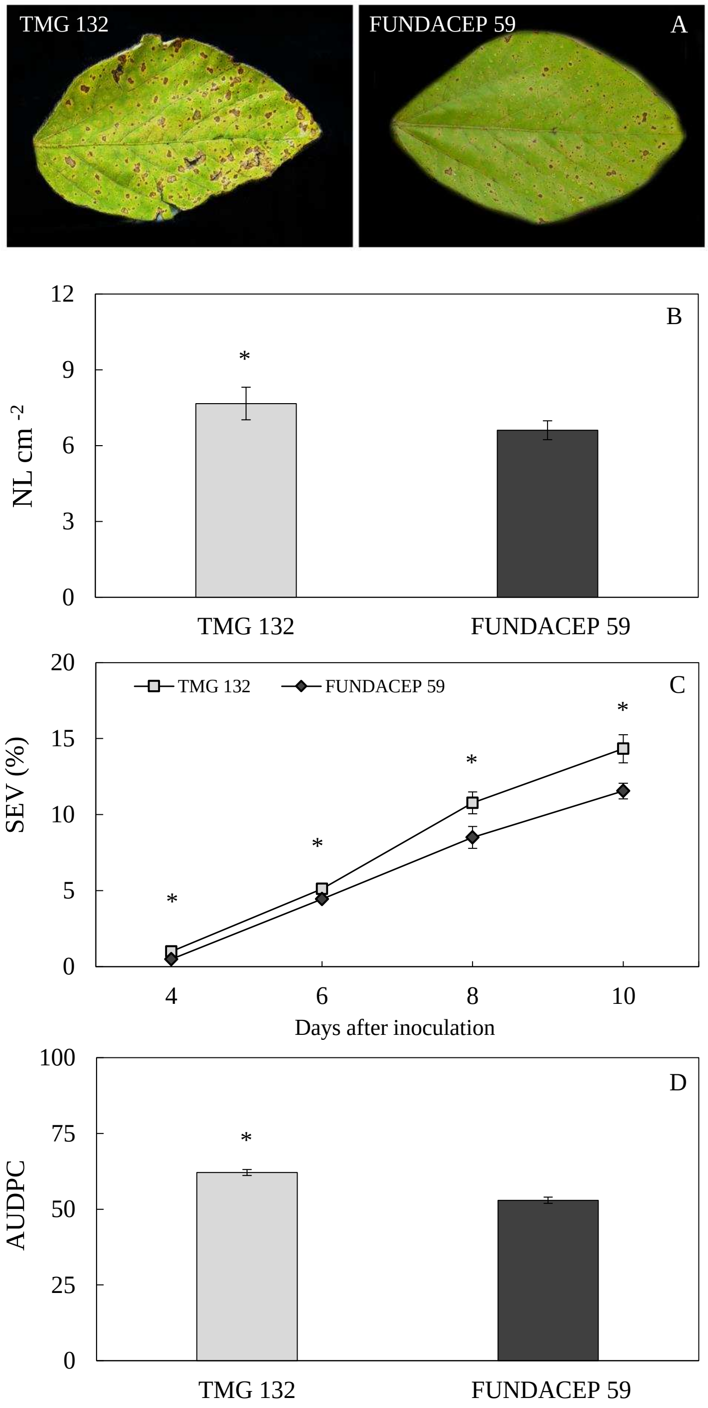
| Variables | C[z] | PI | ST | C × PI | C × ST | PI × ST | C × PI × ST |
|---|---|---|---|---|---|---|---|
| SEV | *** | - | *** | - | *** | - | - |
| NLL | *** | - | - | - | - | - | - |
| AUDPC | *** | - | - | - | - | - | - |
| SOD | ** | *** | *** | ** | ns | ** | ns |
| CAT | ns | *** | ** | ns | ns | ** | ns |
| POX | ** | *** | *** | ** | ns | ** | ns |
| APX | * | *** | *** | ** | ns | ** | ns |
| GPX | ** | *** | ns | ** | ns | ns | ns |
| GR | *** | *** | **z | * | ns | *** | ns |
| GST | ns | *** | *** | ns | ns | *** | * |
| LOX | * | *** | *** | ns | *** | *** | ns |
| AsA | *** | *** | *** | *** | * | ns | ns |
| MDA | ** | *** | ns | ** | ns | ns | ns |
| $H_2O_2$ | ** | *** | ns | ** | ns | ns | ns |
| $O_2^{\bullet -}$ | *** | *** | *** | ** | ns | *** | ns |

[z] Levels of probability: ns = nonsignificant, * = 0.05, ** = 0.01 and *** = < 0.001. - = not determined.

**Table 2** Pearson correlation coefficients among target spot severity (SEV), the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST) and lipoxigenase (LOX) and the concentrations of ascorbate (AsA), malondialdehyde (MDA), hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^{\bullet -}$) in the leaves of soybean plants from cvs. TMG 132 (above the diagonal) and FUNDACEP 59 (below the diagonal) inoculated with *Corynespora cassiicola*.

| Variables | SEV$^{z}$ | SOD | CAT | POX | APX | GPX | GR | GST | LOX | AsA | MDA | $H_2O_2$ | $O_2^{\bullet -}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SEV | - | 0.35 | -0.17 | 0.51$^{*}$ | 0.39 | 0.60$^{*}$ | -0.27 | -0.40 | -0.72$^{**}$ | 0.40 | 0.26 | -0.25 | 0.82$^{***}$ |
| SOD | 0.78$^{***}$ | - | 0.27 | 0.65$^{**}$ | 0.34 | 0.36 | -0.27 | -0.53$^{*}$ | -0.03 | 0.57$^{*}$ | -0.03 | 0.17 | 0.19 |
| CAT | -0.02 | 0.21 | - | 0.32 | 0.04 | 0.49 | -0.53* | -0.13 | 0.56 | 0.28 | -0.15 | -0.15 | -0.40 |
| POX | 0.77$^{***}$ | 0.90$^{***}$ | 0.16 | - | 0.55* | 0.45 | -0.05 | -0.06 | -0.30 | 0.56$^{*}$ | -0.11 | 0.09 | 0.24 |
| APX | 0.63$^{**}$ | 0.54$^{*}$ | -0.10 | 0.67$^{**}$ | - | 0.31 | -0.14 | 0.04 | -0.12 | 0.26 | -0.40 | 0.11 | 0.15 |
| GPX | -0.02 | 0.20 | 0.00 | 0.35 | 0.12 | - | -0.49 | -0.32 | -0.15 | 0.17 | 0.19 | -0.23 | 0.31 |
| GR | -0.31 | -0.18 | 0.09 | -0.07 | 0.32 | 0.08 | - | 0.44 | -0.19 | -0.35 | 0.13 | 0.46 | -0.05 |
| GST | -0.31 | -0.16 | -0.53$^{*}$ | 0.01 | 0.21 | 0.24 | 0.43 | - | 0.10 | -0.48 | -0.05 | 0.39 | -0.53$^{*}$ |
| LOX | -0.77$^{***}$ | -0.80$^{***}$ | -0.06 | -0.72$^{**}$ | -0.61$^{*}$ | -0.07 | -0.18 | 0.09 | - | -0.03 | -0.19 | 0.13 | -0.69$^{**}$ |
| AsA | -0.48 | -0.23 | 0.17 | -0.14 | -0.34 | 0.42 | 0.32 | 0.08 | 0.13 | - | -0.24 | -0.08 | 0.32 |
| MDA | -0.16 | -0.30 | 0.26 | -0.42 | -0.38 | -0.24 | 0.02 | -0.30 | 0.07 | -0.12 | - | 0.24 | 0.27 |
| $H_2O_2$ | -0.01 | 0.02 | -0.18 | 0.05 | 0.03 | 0.27 | 0.09 | 0.13 | -0.14 | 0.19 | 0.12 | - | -0.37 |
| $O_2^{\bullet -}$ | 0.83$^{***}$ | 0.75$^{***}$ | -0.03 | 0.76$^{***}$ | 0.82$^{***}$ | 0.07 | 0.12 | 0.05 | 0.85$^{***}$ | -0.32 | -0.30 | 0.03 | - |

$^{z}$ Levels of probability: $^{ns}$ = not significant, $^{*}$ = 0.05, $^{**}$ = 0.01 and $^{***}$ = < 0.0001.


TMG 132
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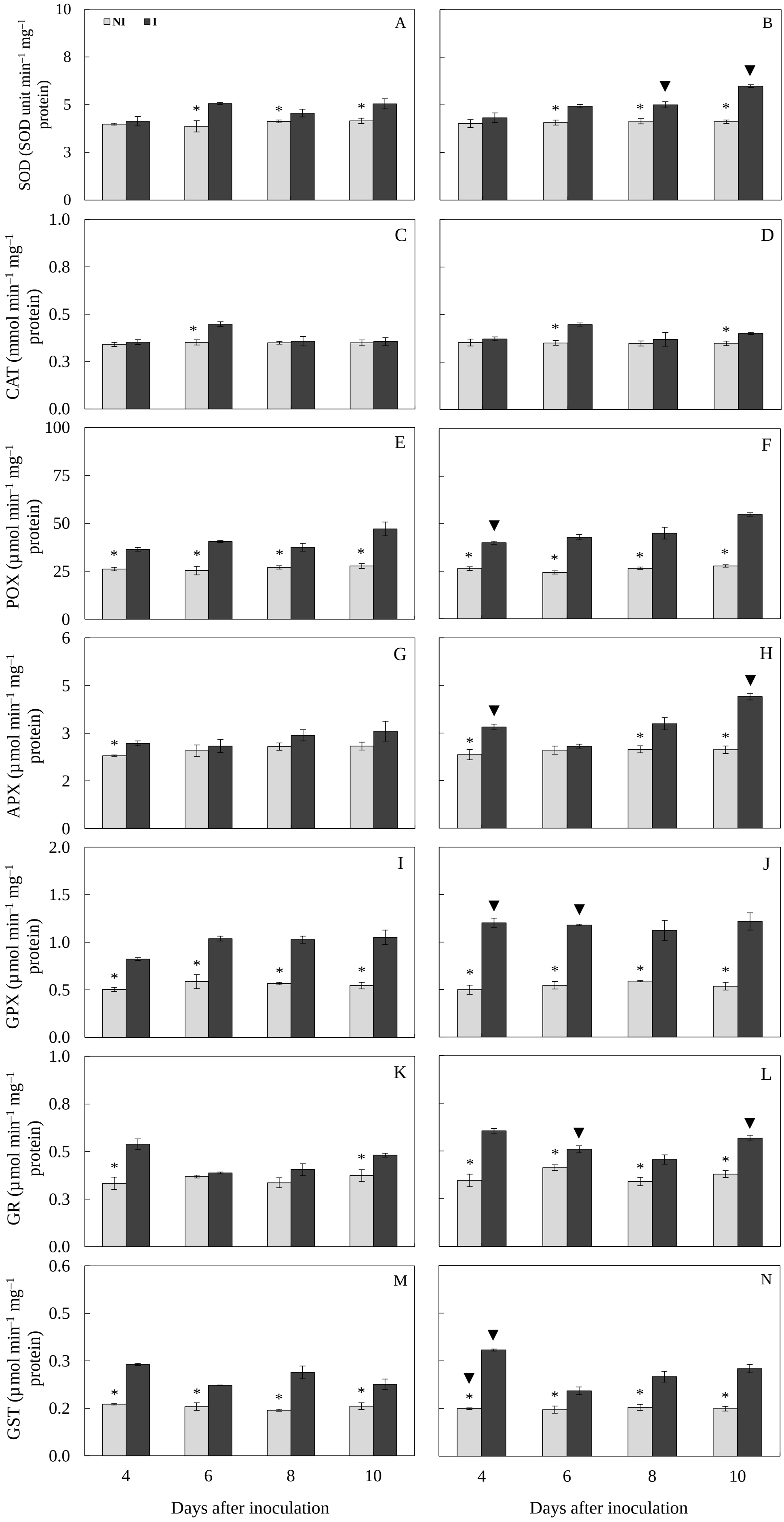
TMG 132
FUNDACEP 59

**Figure 1** Symptoms of target spot (A), number of lesions per leaflet (NLL) (B), target spot severity (SEV) (C) and area under disease progress curve (AUDPC) (D) in the leaves of soybean plants from cvs. TMG 132 and FUNDACEP 59 inoculated with *Corynespora cassiicola*. The means for NLL and AUDPC between the cultivars as well as between the cultivars within each sampling time for SEV 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; results from one representative experiment are shown.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M
N
SOD (SOD unit min⁻¹ mg⁻¹ protein)
CAT (mmol min⁻¹ mg⁻¹ protein)
POX (µmol min⁻¹ mg⁻¹ protein)
APX (µmol min⁻¹ mg⁻¹ protein)
GPX (µmol min⁻¹ mg⁻¹ protein)
GR (µmol min⁻¹ mg⁻¹ protein)
GST (µmol min⁻¹ mg⁻¹ protein)
10
8
5
3
0
1.0
0.8
0.5
0.3
0.0
100
75
50
25
0
6
5
3
2
0
2.0
1.5
1.0
0.5
0.0
1.0
0.8
0.5
0.3
0.0
0.6
0.5
0.3
0.2
0.0
4
6
8
10
Days after inoculation
Days after inoculation
*
▼

**Figure 2** Activities of superoxide dismutase (SOD) (A and B), catalase (CAT) (C and D), peroxidase (POX) (E and F), ascorbate peroxidase (APX) (G and H), glutathione peroxidase (GPX) (I and J), reductase glutathione (GR) (K and L) and glutathione *S*-transferase (GST) (M and N) in the leaves of soybean plants from cvs. TMG 132 (A, C, E, G, I, K and M) and FUNDACEP 59 (B, D, F, H, J, L and N) non-inoculated (NI) or inoculated (I) with *Corynespora cassiicola*. The means between the NI and I treatments within each sampling time as well as between cultivars that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. The means between cultivars within each sampling time and between the NI or I treatments 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; results from one representative experiment are shown.

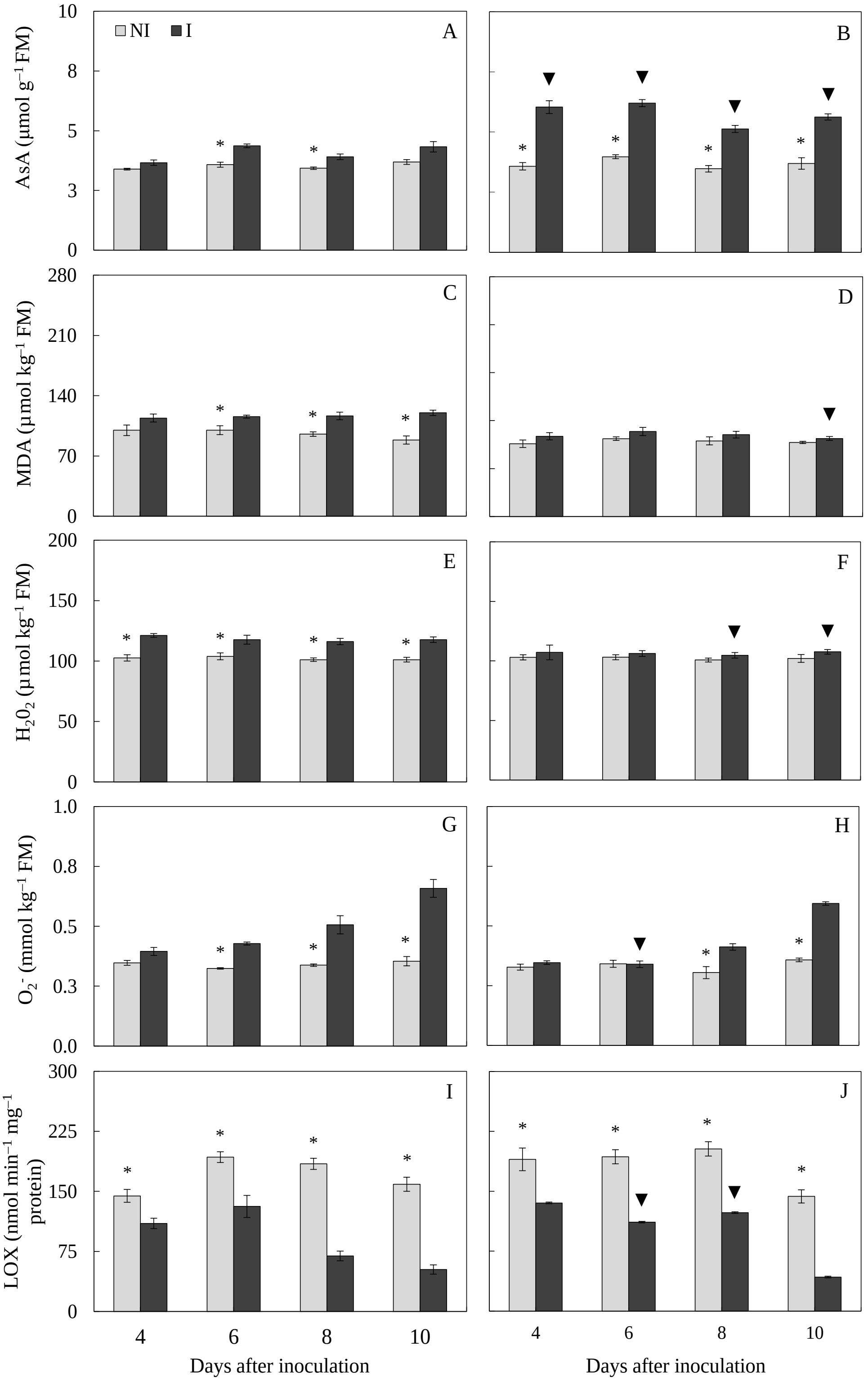
NI
I
A
B
C
D
E
F
G
H
I
J
AsA (µmol g$^{-1}$ FM)
10
8
5
3
0
MDA (µmol kg$^{-1}$ FM)
280
210
140
70
0
$H_2 0_2$ (µmol kg$^{-1}$ FM)
200
150
100
50
0
$O_2^-$ (mmol kg$^{-1}$ FM)
1.0
0.8
0.5
0.3
0.0
LOX (nmol min$^{-1}$ mg$^{-1}$ protein)
300
225
150
75
0
*
▼
4
6
8
10
Days after inoculation
4
6
8
10
Days after inoculation

**Figure 3** Concentrations of ascorbate (AsA) (A and B), malondialdehyde (MDA) (C and D), hydrogen peroxide ($H_2O_2$) (E and F), superoxide ($O_2^{\bullet -}$) (G and H) and lipoxygenase (LOX) (I and J) in the leaves of soybean plants from cvs. TMG 132 (A, C, E, G and I) and FUNDACEP 59 (B, D, F, H and J) non-inoculated (NI) or inoculated (I) with *Corynespora cassiicola*. The means between the NI and I treatments within each sampling time as well as between cultivars that are followed by an asterisk (*) are significantly different ($P \leq 0.05$) by *t*-test. The means between cultivars within each sampling time and between the NI or I treatments 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; results from one representative experiment are shown. FM = fresh matter.

## *CHAPTER 2*

# Defence-related enzymes in the soybean resistance against *Corynespora cassiicola* infection

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## ABSTRACT

Soybean target spot, caused by the fungus *Corynespora cassiicola*, has become a destructive foliar disease on soybean fields in Midwest Brazil. However, information regarding the induction of basal mechanisms of defence in the soybean-*C. cassiicola* interaction has not been investigated. In the present study, the *β*-1-3-glucanase (GLU), chitinase (CHI), phenylalanine ammonia-lyase (PAL), peroxidase (POX), polyphenoloxidase (PPO) activities and the total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives concentrations were studied in soybean leaves from two cultivars (TMG 132 and FUNDACEP 59). There was observed a reducing in the target spot on leaflets of plants from cv. FUNDACEP 59 compared to the leaflets of plants from cv. TMG 132. The activities of GLU, CHI, PAL, POX and PPO significantly increased for the inoculated plants, regardless of cultivar, in comparison to the non-inoculated ones. Significant differences between inoculated plants from both cultivars occurred only for PPO and LTGA concentration at 4 and 6 days after inoculation (dai). For the inoculated plants from cvs. TMG 132 and FUNDACEP 59, there was no difference for the concentration of TSP in comparison to the non-inoculated ones. However, there was a significant increase in TSP for the inoculated plants from cv. FUNDACEP 59 compared to the same plants from cv. TMG 132 counterparts at 4 and 6 dai. The results from the present study indicate the importance of the PPO and the TSP and LTGA derivatives for the soybean resistance to target spot.

Key words: *Corynespora*, foliar disease, *Glycine max*, mechanisms of host defense, target spot.

## INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is one of the most important crops in Brazil and in other countries worldwide (Godoy et al. 2012). However, the occurrence of foliar diseases soybean fields causes significant losses in yield (Yorinori 1997; Sinclair 1999). Target spot, caused by the fungus *Corynespora cassiicola* (Berk and MA Curtis) CT Wei., has become an important foliar disease nowadays on soybean crop in Brazil. Disease symptoms on leaves appear as small lesions with a very well developed yellow halo, which reach two inches in diameter and become circulars and brownish and may coalesce as the disease progress (Almeida et al. 2005; Sinclair 1999). The grown of susceptible cultivars under favorable climatic conditions (*e.g.* high rainfall and high temperatures) cause premature defoliation (Godoy et al. 2012; Teramoto et al. 2013). The fungus also can cause rot in pods and roots, lesions on the stems and eventually reach the seeds (Sinclair 1999). Seed treatment, foliar fungicide spraying and crop rotation are the most used control strategies for target spot management (Almeida et al. 2005). However, due to the absence of cultivars with an adequate level of basal resistance to target spot and the emergency of *C. cassiicola* isolates resistant to fungicides, epidemics of target spot has been frequent in many soybean growing regions in Brazil (Godoy et al. 2012; Teramoto et al. 2013).

As an example of foliar and necrotrophic pathogenesis, *C. cassiicola* completely destroys the leaf cells as a strategy to obtain nutrients, thereby achieving massive growth, and the diffusion of secreted hydrolytic enzymes and non-host selective toxins into the healthy leaf tissues further increases the cellular damage (Lamotte et al. 2006; Onesirosan et al. 1975). Plants respond to pathogens attack through different mechanisms of defense, which include the expression of many defence-related genes, the synthesis of antimicrobial compounds such as phenolics and phytoalexins, the production of reactive oxygen species and tissue lignification (Hückelhoven 2007; Lozovaya et al. 2004; Upchurch and Ramirez 2010). The great capacity of plants to reduce fungal colonization is likely dependent of the combination of these different mechanisms of defense, which greatly change according to the host-pathogen interaction (Hammond-Kosack and Parker 1996; 2003).

For the soybean-*Phakopsora pachyrhizi* interaction, Van de Mortel et al. (2007) reported an early increase in the level of mRNAs transcripts of defense genes on plants from the accession PI230970 carrying the *Rpp2* resistance gene in comparison to a susceptible cultivar. 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). The chitinase and *β*-1,3-glucanase activities were important for mango resistance to floral malformation, caused by *Fusarium mangiferae* (Ebrahima et al. 2011). The genes coding for the enzymes PAL and chalcone synthase and the pathogenesis-related proteins PR1 and PR5 were up-regulated on the roots of plants from a resistant soybean cultivar to *Fusarium solani* f.sp. *glycines* in comparison to the susceptible one (Iqbal et al. 2005). The patterns of genes expression in soybean roots infected by *Phytophthora sojae* showed that lipoxygenase and polyphenol oxidase were strongly down regulated during the time course of pathogen infection (Moy et al. 2004).

Information in the literature regarding how the soybean plants react against *C. cassiicola* infection at the biochemical level is lacking. Therefore, the objective of the present study was to determine some biochemical mechanisms of defense especially the PAL, polyphenol oxidase, chitinase, *β*-1,3-glucanase and peroxidase activities as well as the phenolics and lignin concentrations in two soybean cultivars to *Corynespora cassiicola* infection.

## MATERIAL AND METHODS

### Plant material and growth

A total of ten soybean seeds from cvs. TMG 132 and FUNDACEP 59, susceptible and partially resistant to target spot based on field performance (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 an 1:1:1 mixture of pine bark, peat and expanded vermiculite. Five days after seedlings emergence, each pot was thinned to two seedlings. After the emergence of the cotyledons, plants were fertilized weekly with 50 mL of a nutrient solution, prepared using deionized water, 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 also were watered as needed with deionized water.The plants were kept in greenhouse (relative humidity of 65 ± 5% and temperature of 30 ± 5ºC) during the experiments.

### Inoculation procedure

A pathogenic isolate of *C. cassiicola* obtained from symptomatic soybean plants collected in the city of Rio Verde located in the State of Goiás, Brazil, was used to inoculate the plants. After isolation and morphological identification of conidia, the isolate was preserved by 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 in Petri dishes containing carrot leaf-pea-dextrose-agar (CL-PeDA) media. The CL-PeDA media was prepared using 200 mg carrot leaves, 100 g fresh pea, 20 g dextrose, and 20 g agar, and before media preparation, carrot leaves and peas were mixture in a blender to obtain a homogenous mixture, which was sieved to remove the solids excess. Fragments of fungal mycelia were transferred and homogenously spread to new Petri dishes. The Petri dishes were placed in a growth chamber at 25°C with a 12-h photoperiod for four days. After this period, the fungal colonies were stressed using a Drigalski spatel in a laminar flow chamber to avoid contamination. The plates were then kept in a growth chamber at continous white light (40 W lamps alternately distributed to provide the light intensity of 165.3 $\mu mol\ s^{-1}\ m^{-2}$) for 6 days until the conidia were formed. Conidia were carefully removed from the Petri dishes with a soft-bristle brush using water and 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* ($5 \times 10^4$ conidia $mL^{-1}$) (20 ml per plant). The conidial suspension was applied as a fine mist using a VL Airbrush atomizer (Paache Airbrush Co., Chicago, IL) to both adaxial and abaxial leaf surfaces of each plant until runoff. 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 plant canopy height was ≈ 700 µmol $m^{-2}$ $s^{-1}$ and the temperature was 25 ± 2ºC (day) and 20 ± 2ºC (night). The relative humidity was maintained at 92 ± 3% using a misting system in which nozzles (model NEB-100, KGF Co., São Paulo, Brazil) sprayed mist every 30 min for 15 s above the plant canopy. The temperature and relative humidity were measured with a thermohygrograph (TH-508, Impac, Brazil).

### Disease assessment

Target spot severity was evaluated on the 8th trifoliate leaf from plants of each replication and treatment (cultivars) at 4, 6, 8, and 10 days after inoculation (dai) (based on personal greenhouse trials) using a diagrammatic scale proposed by Soares et al. (2009). Data from target spot severity was used to calculate the area under disease progress curve (AUDPC) according to Shaner and Finney (1977). At 10 dai, the number of lesions per $cm^2$ of leaf area was counted at five randomized places on on the 8th trifoliate leaves from the plants of each replication and treatment with a hand-held microscope.

### Biochemical assay

For all biochemical assays, the 6th, 7th, 8th and 9th trifoliate leaves from the plants of each replication and treatment (cultivars × plant inoculation ) were collected at 4, 6, 8 and 10 dai (based on personal greenhouse trials). Leaf samples were kept in liquid nitrogen during sampling and then stored at -80°C until further analysis.

### Determination of defense enzymes activities

To determine the phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), $\beta$-1,3-glucanase (GLU, EC 3.2.1.6), chitinase (CHI, EC 3.2.1.14), peroxidase (POX, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.10.3.1) activities, a total of 200 mg of leaf tissue was ground into a fine powder with liquid nitrogen in a mortar and pestle. The fine powder was homogenized in 2000 µL 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 collected and used to determine the GLU, CHI, PAL, PPO and POX activities. GLU activity was determined according to method by 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 a 1500 µL of dinitrosalicylic acid (DNS) and incubated at 100°C for 15 min. The reaction was stopped in an ice bath until it 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 (Miller, 1959) and the absorbance was measured at 540 nm. A similar procedure was used to the control samples, but the first incubation was excluded. 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 a 1980 µL of 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 adding 500 µL of 0.2 M sodium carbonate. The control samples had the addition 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 in the extinction coefficient of 70 $mM^{-1}$ $cm^{-1}$. PAL activity was assayed following the methodology proposed by Guo et al. (2007) with some modifications. Primary, the reaction was started by adding 100 µl of crude enzyme extract to 0.9 mL of 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. Therefore, in 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. PAL activity was estimated with an extinction coefficient of 10 $M^{-1}$ $cm^{-1}$ (Zucker 1965). PPO activity was assayed following the colorimetric determination of pyrogallol oxidation according to 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 reaction mixture containing 25 mM potassium phosphate buffer (pH 6.8) and 20 mM pyrogallol. Immediately after reaction had initiated, the absorbance was determined at 420 nm for 1 min at 25ºC. The PPO activity was based in the extinction coefficient of 2.47 $mM^{-1}$ $cm^{-1}$ according to the PPO ionically bounded to the cell wall (Chance and Maehley 1955). The POX activity was determined using the same procedure as described for PPO; therefore the reaction mixture contained 20 mM hydrogen peroxide.

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

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

A total of 100 mg of leaf tissue was ground into a fine powder with liquid nitrogen in a mortar and pestle and homogenized in 1000 µL of a solution containing 80% (v/v) methanol. Next, the crude extract was shaken at 300 rpm at 25°C for 2 h. After, 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 the methanolic extract to 750 µL of 0.2 M Folin-Ciocalteu phenol reagent and incubated at 25ºC for 5 min. The next step was the addition of 0.1 M sodium carbonate to the solution, which was maintained at 25ºC for more 10 min. Afterward, 1000 µL of deionized water also was added to the mixture and incubated at 25ºC during 1 h. The TSP concentration was calculated based on a calibration curve using catechol (Sigma-Aldrich, São Paulo, Brazil) as a standard and the absorbance was read at 725 nm. For the determination of the LTGA derivatives concentration, the pellet was resuspended in 1500 µL of deionized water and homogenized and centrifuged at 12,000 *g* for 15 min. Afterward, the supernatant was discarded and the pellet was dried at 65ºC 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 its concentration was determined by a calibration curve using lignin, alkali and 2-hydroxypropyl ether (Sigma-Aldrich, São Paulo, Brazil) as a standard.

### Experimental design and data analysis

An experiment with 12 replications consisting of two cultivars (TMG 132 and FUNDACEP 59) inoculated with *C. cassiicola* was arranged in a completely randomized design to evaluate the target spot severity, NLL and AUDPC. Another 2 × 2 × 4 factorial experiment with four replications consisting of two cultivars, non-inoculated or inoculated plants and four sampling times with a completely randomized design was used to obtain the samples for the biochemical analysis. For severity, the ANOVA was considered a 2 × 4 factorial experiment consisting of two cultivars and four evaluation times (4, 6, 8 and 10 dai). For NLL and AUDPC, the ANOVA was considered to be an one-way ANOVA consisting of two cultivars. For the biochemical variables, the ANOVA was considered a 2 × 2 × 4 factorial consisting of two cultivars, plant inoculation (non-inoculated and inoculated plants) and four sampling times (4, 6, 8 and 10 dai). Each experimental unit was consisted of a 2 L plastic pot with two plants. Data from all variables were analyzed by analysis of variance (ANOVA) and means from the treatments were compared using *t*-tests ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). The Pearson linear correlation technique was used to determine the relationships among target spot severity and the GLU, CHI, PAL, POX and PPO activities as well as the concentrations of TSP and LTGA derivatives.

## RESULTS

For NLL and AUDPC, the factor cultivars was significant (Table 1). The factors cultivars and sampling time as well as their interaction were significant for target spot severity (Table 1). The target spot symptoms, characterized as circular brownish lesions with yellow halos, were more evident on the leaflets of plants from cv. TMG 132 in comparison to the leaflets of plants from cv. FUNDACEP 59 (Fig. 1a). The target spot severity significantly decreased by 50, 22, 17 and 25% at 4, 6, 8 and 10 dai, respectively, on the leaflets of plants from cv. TMG 132 in comparison to the leaflets of plants from cv. FUNDACEP 59 (Fig. 1c). There was observed a reduction of NLL and AUDPC by 26 and 16%, respectively, in cv. FUNDACEP 59 compared to the same plants from cv. TMG 132 (Fig. 1C and 1D).

For GLU and PAL activities, only the factor plant inoculation was significant. The factors plant inoculation and sampling time were significant for CHI, POX and PPO activities and for the TSP concentration. For the LTGA derivatives concentration, only the factors cultivars and sampling time was significant (Table 1). Only the interaction plant inoculation × sampling time was significant for GLU, POX and PPO activities and for the concentration of TSP (Table 1). For cvs. TMG 32 and FUNDACEP 59, GLU, CHI, PAL, POX and PPO activities significantly increased for the inoculated plants in comparison to the 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). After inoculation, there was no difference in TSP concentration, regardless of cultivar, compared to the non-inoculated ones (Fig. 3). The LTGA derivatives concentration significantly increased from 4 to 10 dai for the inoculated plants in comparison to the non-inoculated counterparts regardless of the cultivar (Fig. 3). However, in inoculated plants from cv. FUNDACEP 59, there was recorded a significant increase of TSP and LTGA derivatives concentration at early stages of fungal infection compared to the same plants from cv. TMG 132 (Fig. 3).

For cv. TMG 132, there was a positive correlation between target spot severity with POX and PPO activities and the LTGA derivatives concentration (Table 2). For cv. FUNDACEP 59, there was a positive correlation between target spot severity and POX activity and between PAL activity and the LTGA derivatives concentration (Table 2).

## DISCUSSION

The results from the present study provide novel biochemical evidences that an increase in the basal resistance of soybean plants from two cultivars differing in response to *C. cassiicola* infection was able to reduce target spot development. The reduced target spot severity, NLL and AUDPC on the leaves of plants from cv. FUNDACEP 59 compared to plants from cv. TMG 132 confirmed their differencial response in terms of basal resistance to *C. cassiicola* infection previous reported on field performance (Godoy et al. 2012; Pitol et al. 2011). Increases in the activities of the defence enzymes studied as well as on the concentrations of TSP and LTGA derivatives in this study corroborated with previous reports in the literature(Gnanamangai et al. 2011; Leite et al. 2014; Li and Steffens 2002; Lozovaya et al. 2004; Roulin and Buchala 1995; Sahoo et al. 2009; Siranidou et al. 2002).

In plants, the constitutive CHI and GLU activities is low, but they dramatically increase under abiotic and biotic stress (Wu and Bradford 2003). Accordingly, higher CHI and GLU activities were observed for the inoculated plants from cvs. TMG 32 and FUNDACEP 59 in comparison to the non-inoculated counterparts. Similarly, Ebrahima et al. (2011) showed that during *Fusarium mangiferae* infection in mango plants, the CHI and GLU activities were significantly increased in response to fungal infection. CHI and GLU are important enzymes involved in host defense against pathogen attack because they hydrolyze the major components of cell wall of several fungi, chitin and *β*-1,3-glucan, respectively (Gnanamangai et al. 2011; Lattanzio et al. 2006). CHI and GLU activities showed a similar pattern during the time course of fungal infection on plants from both cultivars. Similarly, in potato plants from susceptible and resistant cultivars, there was a strong and coordinated increase in the CHI and GLU activities in response to *Phytophthora infestans* infection (Schröder et al. 1992). However, in infected leaves of *Camellia sinensis*, only the CHI activity was higher in response to *Cercospora theae* infection (Gnanamangai et al. 2011). Although the CHI and GLU activities in the leaves of plants from cvs. TMG 132 and FUNDACEP 59 were similar, they were important, together with the other enzymes studied, to reduce the target spot symptoms especially on plants from cv. FUNDACEP 59.

In the present study, PAL activity on inoculated leaves was higher than for the non-inoculated plants regardless of cultivar. This increase is of great importance for soybean resistance to target spot because PAL is the major enzyme in the phenylpropanoid pathway that catalyzes the deamination of *L*-phenylalanine for the synthesis of various phenolic compounds with antimicrobial activity, signaling molecules and lignin biosynthesis (Borges et al. 2012; Dixon et al. 2002; Hao et al. 2011). Accordingly, Upchurch and Ramirez (2010) showed a significant up-regulation of the *PAL* gene on soybean detached leaves and seeds after infection by *Cercospora kikuchii* and *Diaporthe phaseolorum* var. *meridionalis*. Although the PAL activity in the inoculated plants was higher than for the non-inoculated regardless of cultivars, there was no difference for its activity for inoculated plants from cvs. TMG 132 and FUNDACEP 59. By contrast, the PAL activity on the roots from a partially resistant cultivar of *Capsicum annuum* was higher than for the susceptible one after inoculation with *Phytophthora capsici* (Zhang et al. 2013). The gene coding for the PAL enzyme was up-regulated on the roots of plants from a resistant soybean cultivar to *F. solani* f.sp. *glycines* in comparison to a susceptible one (Iqbal et al. 2005). Although PAL activity on the leaves of plants from cv. FUNDACEP 59 was similar to those from cv. TMG 132, its participation in the phenylpropanoid pathway for cv. FUNDACEP 59 probably was determinant to the increase in the biosynthesis of lignin that promoted the strengthening of plant cell walls.

POX plays a key role in the host defense response through its participation in the production of antimicrobial quantities of hydrogen peroxide to be used for cell wall lignification or cross-linking with the cell wall proteins (Chittoor et al. 1999; Hiraga et al. 2001; Torres et al. 2006). POX activity on the leaves of plants from cvs. TMG 132 and FUNDACEP 59 increased in response to *C. cassiicola* infection. By contrast, in response to *Phytophthora sojae*, *POX* gene was among those most strongly down-regulated in soybean roots during the course of the oomycete infection (Moy et al. 2004). Conversely, in pepper plants, POX activity in the resistant and partially resistant cultivars increased in response to *P. capsici* infection compared to the susceptible one (Zhang et al. 2013). Similarly, Leite et al. (2014) reported that POX activity was higher in inoculated plants from a resistant genotype than for the susceptible one. However, in the present study, no difference between cultivars for POX activity upon *C. cassiicola* was detected.

Upon *C. cassiicola* infection, PPO activity increased on the leaves of plants from cvs. TMG 132 and FUNDACEP. Increased levels of phenolics provides an adequate substrate to oxidative reactions catalyzed by PPO, which consume oxygen and produce fungitoxic quinones inside plant tissues, making the medium unfavorable to the further development of pathogens (Lattanzio et al. 2006). Accordingly, increases in PPO activity were associated with raises in quinone levels, which, in turn, negatively affected the growth of *Phlyctaena vagabunda* (Lattanzio et al. 2001). Furthermore, Upchurch and Ramirez (2010) showed an up regulation of the *PPO* gene on soybean detached leaves and seeds infected by *C. kikuchii* or *D. phaseolorum* var. *meridionalis* compared to the non-infected leaves and seeds. Changes in phenolics concentration and PPO activity may be considered as a part of the plant response against pathogens infection, which arrest fungal cells avoiding further damage to the healthy surrounding tissues (Lattanzio et al. 2006). Indeed, transgenic tomato plants overexpressing the *PPO* gene showed reduced bacterial speck severity and a strong inhibition of *Pseudomonas syringae* pv. *tomato* growth compared to the wild type plants (Li and Steffens 2002). In the present study, inoculated plants from cv. FUNDACEP 59 showed an increase in PPO activity, especially at the early stages of fungal infection and less disease symptoms in comparison to the plants from cv. TMG 132. In agreement with this finding, Sahoo et al. (2009) reported an increase in the phenolics concentration and PPO activity on the leaves of plants from resistant genotypes of taro in comparison to inoculated plants from susceptible genotype after inoculation with *Phytophthora colocasiae*. The increases in PPO activity and LTGA derivatives on the leaves of plants from cv. FUNDACEP 59 could explain the reduced target spot severity in this cultivar in comparison with the susceptible one.

TSP can act negatively affecting the pathogen infection by increasing its membrane permeability (Tomás-Barberán et al. 1990) and inhibiting secreted pathogenic lytic enzymes (Lattanzio et al. 2006). In the present study, there was no difference in the TSP concentration for the inoculated plants, regardless of cultivar, compared to the non-inoculated counterparts. However, Polanco et al. (2012) showed that for the bean-*Colletotrichum lindemuthianum* interaction, the reduction in the TSP concentration was associated to the increased production of lignin precursors determined as LTGA derivatives. As showed in the present study, the unchanged TSP concentration for the inoculated plants from both cultivars compared to the control plants can be linked to its conversion to LTGA derivatives. However, the higher TSP concentration observed for the inoculated plants from cv. FUNDACEP 59 compared to the plants from cv. TMG 132 could explain the increases in the LTGA derivatives concentration for this cultivar during *C. cassiicola* infection. Accordingly, Leite et al. (2014) showed that the increase in the concentration of lignin for the inoculated plants from a resistant cultivar compared to a susceptible one contributed to the resistance of bean plants to *Sclerotinia sclerotiorum*. Moreover, higher lignin concentration was observed in the inoculated roots of soybean plants from resistant lines compared to the susceptible ones in response to *F. solani* f.sp. *glycines* infection. It has been hypothesized that the deposition of lignin interferes with enzymatic hydrolysis and mechanical penetration of plant tissue by fungal pathogens interfering with the movement of water and diffusible molecules as non-selective toxins in the interface plant and fungus (Lattanzio et al. 2006; Siranidou et al. 2002). The synthesis and delivery of a non-selective toxin named cassiicolin into soybean host tissues can greatly contribute to the aggressiveness of many *C. cassiicola* isolates (Barthe et al. 2007; Lamotte et al. 2006; Onesirosan et al. 1975; Passos et al. 2010). It seems plausible to assume that a higher LATG derivatives concentration on the leaves of plants from cv. FUNDACEP 59 probably contributed to the reduction of the deleterious effects of cassiicolin.

In conclusion, the results of the present study clearly demonstrate that the basal level of resistance was increased upon *C. cassiicola* infection. In this scenario, the early increase in PPO activity and the TSP and LTGA derivatives concentration were important for the soybean resistance to target spot, especially for the cv. FUNDACEP 59.

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## LIST OF TABLES AND FIGURES

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

| Variables | $C^z$ | PI | ST | C × PI | C × ST | PI × ST | C × PI × ST |
|---|---|---|---|---|---|---|---|
| SEV | *** | - | *** | - | *** | - | - |
| NLL | *** | - | - | - | - | - | - |
| 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 |
| TSP | ns | *** | *** | ns | ns | *** | ns |
| LTGA | ** | ns | ** | ns | ns | ns | ns |

$^{z}$ Levels of probability: $^{ns}$ = nonsignificant, $^{**}$ = 0.01 and$^{***}$ = < 0.001. - = not determined.

**Table 2** Pearson correlation coefficients among target spot severity (SEV), the activities of $\beta$-1-3-glucanases (GLU), chitinases (CHI), phenylalanine ammonia-lyases (PAL), peroxidases (POX), polyphenoloxidases (PPO) and the concentrations of total soluble phenolics (TSP) and lignin-thioglycolic acid (LTGA) derivatives in the leaves of soybean plants from cvs. TMG 132 and FUNDACEP 59 inoculated with *Corynespora cassiicola*.

| Variables | SEV[z] | GLU | CHI | PAL | POX | PPO | TSP | LTGA |
|---|---|---|---|---|---|---|---|---|
| SEV | - | $0.47^{ns}$ | $0.02^{ns}$ | $0.01^{ns}$ | $0.61^{*}$ | $0.63^{**}$ | $0.47^{ns}$ | $0.55^{*}$ |
| GLU | $0.16^{ns}$ | - | $0.08^{ns}$ | $0.29^{ns}$ | $0.26^{ns}$ | $0.43^{ns}$ | $0.12^{ns}$ | $-0.08^{ns}$ |
| CHI | $-0.03^{ns}$ | $0.33^{ns}$ | - | $0.36^{ns}$ | $0.19^{ns}$ | $-0.09^{ns}$ | $0.03^{ns}$ | $-0.11^{ns}$ |
| PAL | $-0.25^{ns}$ | $-0.23^{ns}$ | $-0.22^{ns}$ | - | $-0.38^{ns}$ | $-0.32^{ns}$ | $0.27^{ns}$ | $-0.18^{ns}$ |
| POX | $0.58^{*}$ | $0.16^{ns}$ | $0.15^{ns}$ | $0.13^{ns}$ | - | $0.42^{ns}$ | $0.24^{ns}$ | $0.27^{ns}$ |
| PPO | $0.46^{ns}$ | $0.34^{ns}$ | $0.03^{ns}$ | $-0.34^{ns}$ | $0.27^{ns}$ | - | $0.12^{ns}$ | $0.22^{ns}$ |
| TSP | $-0.22^{ns}$ | $-0.07^{ns}$ | $0.41^{ns}$ | $-0.02^{ns}$ | $-0.16^{ns}$ | $-0.40^{ns}$ | - | 0.03 |
| LTGA | $-0.35^{ns}$ | $-0.05^{ns}$ | $-0.15^{ns}$ | $0.73^{**}$ | $-0.06^{ns}$ | $-0.31^{ns}$ | $-0.11^{ns}$ | - |

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

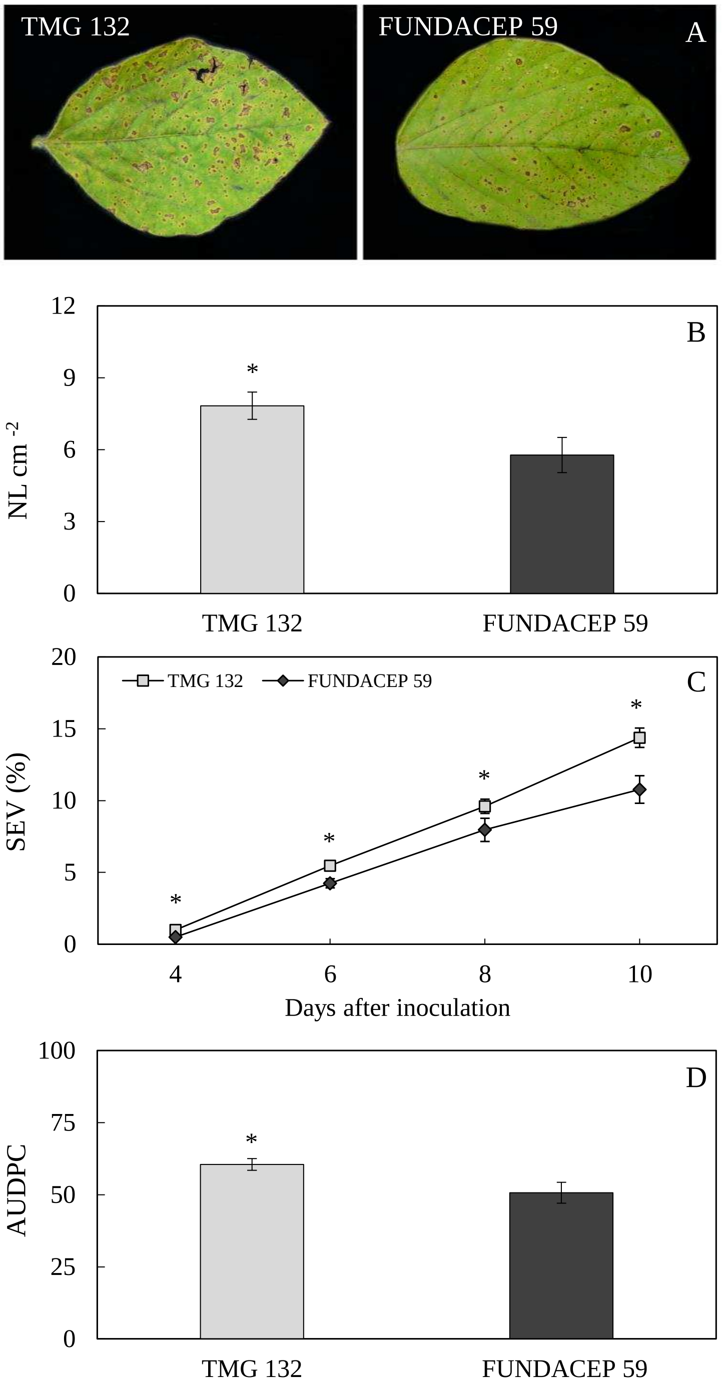
TMG 132
FUNDACEP 59
A
B
12
9
6
3
0
NL cm -2
*
TMG 132
FUNDACEP 59
C
20
15
10
5
0
SEV (%)
TMG 132
FUNDACEP 59
*
*
*
*
4
6
8
10
Days after inoculation
D
100
75
50
25
0
AUDPC
*
TMG 132
FUNDACEP 59

**Fig. 1** Symptoms of target spot (A), number of lesions per leaflet (NLL) (B), target spot severity (SEV) (C) and area under disease progress curve (AUDPC) (D) for soybean plants from cvs. TMG 132 and FUNDACEP 59 inoculated with *Corynespora cassiicola*. The means for either NLL or AUDPC between the cultivars as well as between the cultivars within each sampling time for SEV 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; results from one representative experiment are shown.

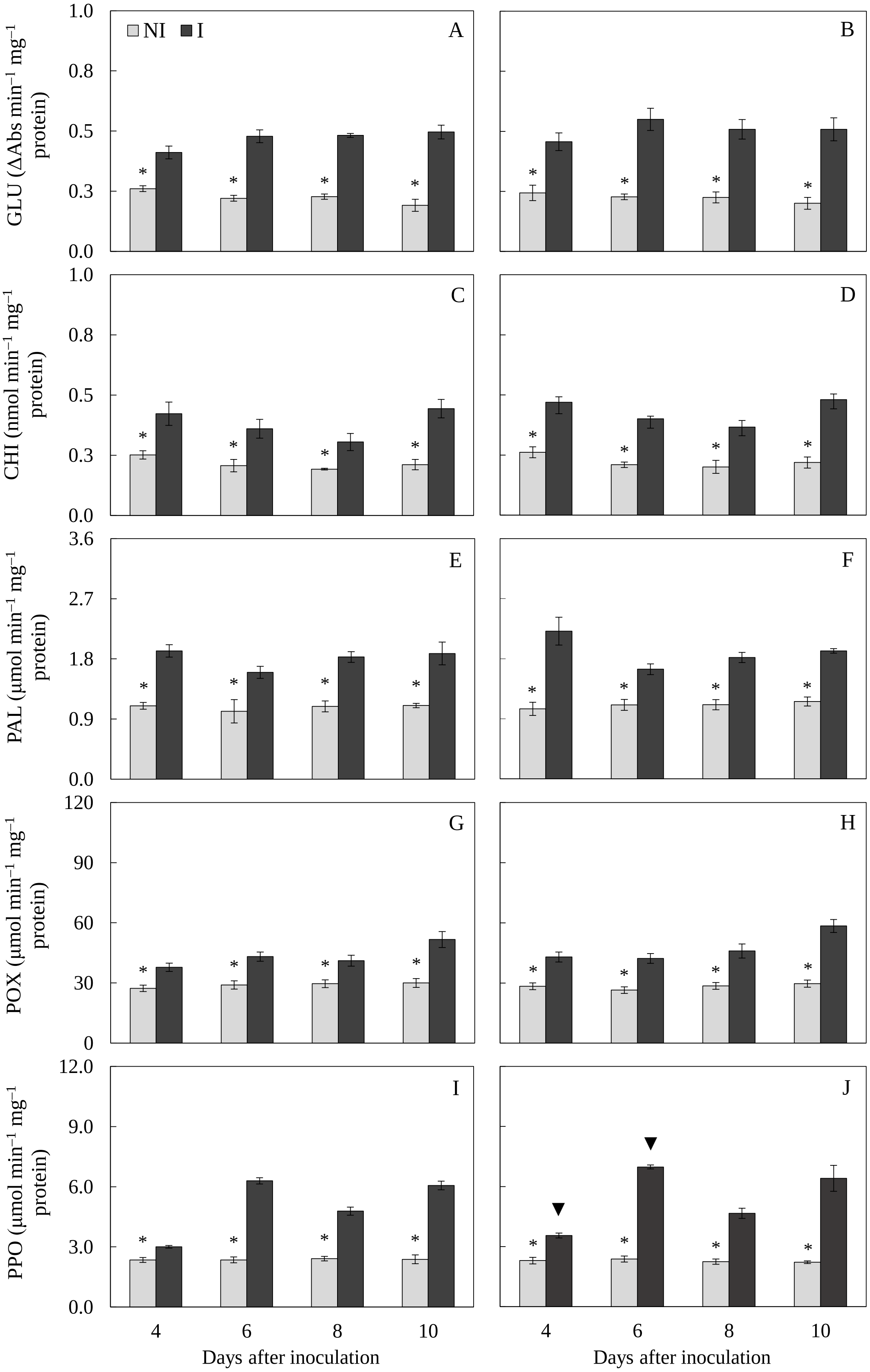
NI
I
A
B
C
D
E
F
G
H
I
J
GLU (ΔAbs min⁻¹ mg⁻¹ protein)
CHI (nmol min⁻¹ mg⁻¹ protein)
PAL (µmol min⁻¹ mg⁻¹ protein)
POX (µmol min⁻¹ mg⁻¹ protein)
PPO (µmol min⁻¹ mg⁻¹ protein)
1.0
0.8
0.5
0.3
0.0
3.6
2.7
1.8
0.9
120
90
60
30
0
12.0
9.0
6.0
3.0
4
6
8
10
Days after inoculation
*
▼

**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), polyphenoloxidases (PPO) (I and J) in the leaves of soybean plants from cvs. TMG 132 (A, C, E, G and I) and FUNDACEP 59 (B, D, F, H and J) non-inoculated (NI) or inoculated (I) with *Corynespora cassiicola*. For each cultivar, 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 NI or 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; results from one representative experiment are shown.

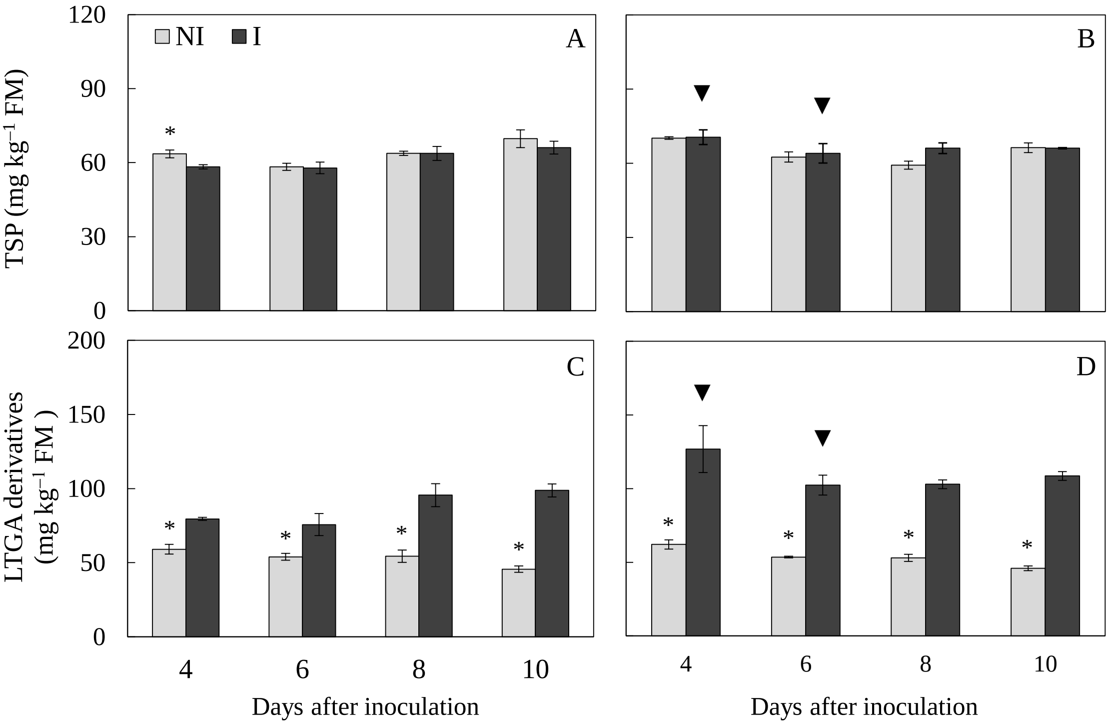

**Fig. 3** Concentrations of total soluble phenolics (TSP) (A and B) and lignin-thioglycolic acid (LTGA) derivatives (C and D) in the leaves of soybean plants from cvs. TMG 132 (A and C) and FUNDACEP 59 (B and D) non-inoculated (NI) or inoculated (I) with *Corynespora cassiicola*. For each cultivar, 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 NI or 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; results from one representative experiment are shown. FM = fresh matter.

# GENERAL CONCLUSIONS

1. The infection of soybean plants by *C. cassiicola* triggered lipid peroxidation via production of $O_2^{\bullet -}$ and $H_2O_2$, thus leading to the induction of antioxidative mechanisms of soybean, regardless of cultivar resistance.
2. The greater increases on SOD, POX, APX, GPX, GR and GST the activities and on the concentration of AsA for plants from cultivar FUNDACEP 59 compared to plants from cultivar TMG 132 helped to reduce the concentrations of MDA, $H_2O_2$, and $O_2^{\bullet -}$, especially in the former cultivar.
3. The *C. cassiicola* infection triggered increases in the activities of CHI, GLU, PAL, POX and PPO, regardless of cultivar, showing which the increase of these enzymes may be a defense strategy of soybean plants against *C. cassiicola* infection.
4. High PPO activity associated with greater concentrations of TSP and LTGA derivatives for plants from cultivar FUNDACEP 59 contributed to the increase in resistance to target spot.