Changes in the Antioxidant System in Soybean Leaves Infected by *Corynespora cassiicola*

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ABSTRACT

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Considering the importance of target spot, caused by the fungus *Corynespora cassiicola*, to reduce soybean yield in Brazil and that more basic information regarding the soybean–*C. cassiicola* interaction is needed, the present study aimed to investigate whether the cellular damage caused by *C. cassiicola* infection could activate the antioxidant system and whether a more efficient antioxidant system could be associated with an increase in soybean resistance to target spot. The activities of the antioxidant enzymes superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione peroxidase, glutathione reductase, glutathione S-transferase as well as the concentrations of ascorbate (AsA), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and malondialdehyde (MDA) were measured in soybean plants from two cultivars differing in resistance to the pathogen. The number of lesions per square centimeter was significantly reduced by 14% in plants from cultivar Fundacep 59 compared with plants from cultivar TMG 132. The area under the disease progress curve was significantly lower, by 15%, in plants from Fundacep 59 than in plants from TMG 132. Generally, antioxidant enzyme activities and AsA concentration significantly increased in response to *C. cassiicola* infection in plants from both cultivars, however more prominent increases were recorded for plants from Fundacep 59. The concentrations of MDA, H_2O_2, and $O_2^{\bullet -}$ also increased, particularly for plants from TMG 132. The results from this study highlight the importance of a more efficient antioxidative system in the removal of reactive oxygen species generated in soybean plants during *C. cassiicola* infection, contributing to the resistance to target spot.

Soybean (*Glycine max* (L.) Merrill) is one of the most profitable crops grown worldwide, but many fungal diseases greatly contribute to reduced grain quality and yield (Sinclair 1999). In Brazil, target spot, which is caused by the necrotrophic fungus *Corynespora cassiicola* (Berk and MA Curtis) CT Wei., has become increasingly important (Godoy et al. 2012; Teramoto et al. 2013). Symptoms consisted of roughly circular, necrotic leaf lesions from minute to 11 mm in diameter, though typically approximately 4 to 5 mm in diameter, and with a yellow margin (Sinclair 1999). The necrotic lesions occasionally are large and are of roughly circular to irregular form, 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 can exhibit a zonate pattern that significantly decreases the photosynthetically active leaf area (Sinclair 1999), and premature defoliation may occur in susceptible cultivars (Almeida et al. 2005; Sinclair 1999). The fungus survives between seasons on soybean debris and seed (Almeida et al. 2005; Sinclair 1999). Elevated temperatures (28 ± 2°C) associated with high relative humidity in the mid-to-late soybean growing season, especially when the plant canopy has closed, favor the occurrence of severe target spot epidemics (Godoy et al. 2012). Target spot management has been difficult to achieve because few cultivars have a desirable level of resistance and isolates of *C. cassiicola* resistant to the benzimidazole fungicides have recently been detected in Brazil (Teramoto et al. 2013; Xavier et al. 2013).

Because *C. cassiicola* is able to secrete many lytic enzymes and non-host-selective toxins, such as cassiicolin, the fungus can use these macromolecules during its process of pathogenesis for a massive colonization of host's tissue (Barthe et al. 2007; Onesirosan et al. 1975b). The reactive oxygen species (ROS) have been associated with plant defense due to their antimicrobial activity or their contribution to the lignification of host cell walls that constrains pathogen colonization of the host tissue (Daub et al. 2013; Grant and Loake 2000). However, ROS are strong oxidizing agents that can damage essential macromolecules, such as membrane lipids, pigments, nucleic acids, carbohydrates, and proteins, and therefore contribute to premature leaf senescence (Gill and Tuteja 2010). To maintain an adequate balance between the production and removal of ROS, plants have evolved an antioxidant system (Mittler 2002). This system involves a wide range of compounds, including reduced ascorbate (AsA) and glutathione (GSH), carotenoids, fiavonoids, and other phenolics in addition to miscellaneous antioxidant enzymes, which include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione-*S*-transferase (GST), and glutathione reductase (GR) (Asada 1999; Malenčić et al. 2010; Mittler et al. 2004; Mittler 2002; Noctor and Foyer 1998). During the pathogenesis of the necrotrophic fungus *Botrytis cinerea* in tomato plants, peroxisomal CAT activity increased in the infected plants during the first stages of fungal infection, but it strongly declined during the advanced stages compared with healthy plants (Kuźniak and Skłodowska 2005). Increases in the activities of antioxidant SOD, POX, and reduced glutathione during fungal infection were associated with the removal of ROS in the soybean–*Sclerotinia sclerotiorum* interaction (Malenčić et al. 2010). Soybean seedlings infected by *Rhizoctonia solani* also exhibited a significant increase in SOD activity compared with noninfected seedlings (Kiprovski et al. 2012). During *Pyricularia oryzae* infection in wheat plants, Debona et al. (2012) showed significant increases in SOD, CAT, POX, APX, GST, and GR activities in plants from a resistant cultivar in comparison with plants from a susceptible one. In agreement with

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previous information of the efficiency of the removal of ROS during pathogenesis on resistant plants, Lanubile et al. 2012 observed increases in SOD activity in maize ears from resistant cultivar infected by *Fusarium verticillioides* compared with the susceptible one.

Considering the importance of target spot to soybean production and that more basic information regarding the soybean–*C. cassiicola* interaction is needed, the present study tested the following hypotheses: (i) *C. cassiicola* infection of soybean plants increases ROS production and cellular damage, activating the antioxidant system, and (ii) a more efficient antioxidant system is associated with an increase in soybean resistance to target spot.

MATERIALS AND METHODS

Soybean growth. A total of 10 soybean seed from TMG 132 and Fundacep 59, susceptible and moderately resistant to target spot, respectively (Godoy et al. 2012; Pitol et al. 2011), were sown into 2 liter plastic pots (Ecovaso, Jaguariúna, SP, Brazil) containing 2 kg of Tropstrato (Vida Verde, Mogi Mirim, SP, Brazil) substrate composed of a 1:1:1 mixture of pine bark, peat, and expanded vermiculite. Five days after seedling emergence, each pot was thinned to two seedlings, which were fertilized weekly with 50 ml of a nutrient solution prepared using deionized water that contained 40 mM KNO_3, 10 mM $NH_4H_2PO_4$, 10 mM $MgSO_4{\cdot}7H_2O$, 15 mM $Ca(NO_3)_2{\cdot}4H_2O$, 2.4 mM $ZnSO_4{\cdot}7H_2O$, 3 mM H_3BO_3, 10 mM K_2SO_4, 3.3 mM CH_4N_2O, and 7.5 mM $NH_4H_2SO_4$ (Dallagnol et al. 2012). Plants were watered with deionized water as needed. The plants were kept in a greenhouse (relative humidity of 65 ± 5% and temperature of 30 ± 5°C) and were grown for 45 days (approximately V9 growth stage) (Fehr et al. 1971).

Inoculation procedure. A pathogenic isolate of *C. cassiicola*, obtained from symptomatic leaves of soybean plants collected in the municipality of Rio Verde, Goiás State, Brazil, was used to inoculate the plants. The obtained *C. cassiicola* isolate was preserved using Castellani's method, which consisted of five plugs containing the PDA media and mycelium (5 mm diameter) being placed in a flask containing 5 ml of distilled sterilized water (Dhingra and Sinclair 1995). At 14 days before inoculation, plugs of mycelium from cultures grown on potato dextrose agar were placed in Petri dishes containing carrot leaf-pea dextrose agar (CL-PeDA) media. The CL-PeDA media was prepared using 200 mg of carrot leaves, 100 g of fresh peas, 20 g dextrose, and 20 g of agar. Carrot leaves and peas were mixed in a blender to obtain a homogenous mixture, which was then sieved to remove the excess solids. Fragments of 3-day-old fungal mycelia were transferred and homogenously spread onto a fresh plate of CL-PeDA and placed in a growth chamber with a 12-h photoperiod at 25°C for 4 days. After this period, the fungal mycelia were stressed using a Drigalski spatula in a laminar flow chamber to avoid contamination (Onesirosan et al. 1975a). The plates were subsequently maintained in a growth chamber under continuous white light (40 W lamps alternately distributed to provide a light intensity of 165.3 μmol s^{-1} m^{-2}) for 6 days until the production of conidia. Conidia were carefully removed from Petri dishes with a soft-bristle brush using water containing gelatin (1% wt/vol). The plants were grown at greenhouse conditions (25 ± 2°C during the day and 20 ± 2°C at night) prior to inoculation. The conidial suspension of *C. cassiicola* was prepared by adding approximately 30 ml of water in each plate (10 cm diameter) with abundant sporulation, and then brushing to remove the conidia from the mycelium resulting in a concentrated suspension that was calibrated to a final concentration of 5×10^4 conidia ml^{-1}. A total of 20 ml of conidial suspension was applied as a fine mist using a VL airbrush atomizer (Paache Airbrush Co., Chicago, IL) to both the adaxial and abaxial leaf surfaces of each plant. After inoculation, plants were maintained in a plastic mist growth chamber (MGC) inside a greenhouse for the duration of the experiments. The MGC was constructed of wood (2 m wide, 1.5 m high, and 5 m long) and covered with transparent plastic (100 μm thick). The maximum natural photon flux density at plant canopy height was ≈700 μmol m^{-2} s^{-1} and the temperature was 25 ± 2°C during the day and 20 ± 2°C at night. The relative humidity was maintained at 90 ± 5% using a misting system that sprayed mist from nozzles (model NEB-100, KGF Co., São Paulo, Brazil) above the plant canopy for 15 s every 30 min. The temperature and relative humidity were measured with a thermohygrograph (TH-508, Impac, Brazil).

Disease assessment. Target spot severity (TSS) on the eighth trifoliate leaf was evaluated for plants from each replication and treatment at 4, 6, 8, and 10 days after inoculation (dai) using a standard area diagram set (Soares et al. 2009). The TSS data were used to calculate the area under the disease progress curve (AUDPC) according to Shaner and Finney (1977). At 10 dai, the number of lesions (NL) per square centimeter of leaflet area was counted at five random places on the eighth trifoliate leaves with the aid of a hand-held microscope (×15).

Biochemical analysis. For all biochemical assays, the sixth, seventh, eighth, and ninth trifoliate leaves, counted from the base to the top, of plants from each treatment and replication were collected at 4, 6, 8, and 10 dai. Leaf samples were kept in liquid nitrogen during sampling and subsequently stored at –80°C until further analysis.

Determination of enzyme activities. To determine the activities of SOD, CAT, POX, APX, GPX, and GST, a total of 200 mg of homogenized leaf tissue was ground into a fine powder using a mortar and pestle while adding liquid nitrogen. The fine powder was immediately homogenized in 2 ml of a solution containing 50 mM of a potassium phosphate buffer (pH 6.8), 0.1 mM ethylenediaminetetraacetic (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2% (wt/vol) polyvinylpolypyrrolidone (PVPP). The homogenate was then centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatant was used as a crude enzyme extract. To determine the activity of glutathione reductase (GR), a total of 200 mg of homogenized leaf tissue was ground as described above, and the extraction solution used to homogenize the fine powder contained 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 1 mM *DL*-dithiothreitol, 1 mM PMSF, and 2% (wt/vol) PVPP in a final volume of 2 ml. The homogenate was centrifuged as previously described.

The SOD activity was determined using the method described by Del Longo et al. (1993), which measures the capacity of SOD to photochemically reduce the *p*-nitrotetrazolium blue (NTB) in the reaction solution. The reaction was initiated following 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 15W lamp light per 10 min. Following 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 with a spectrophotometer (Evolution 60, Thermo Fisher Scientific Inc., MA) (Giannopolitis and Ries 1977). For the control samples, the reaction mixture was maintained in darkness for 10 min, and the absorbance was measured at 560 nm. The values obtained from the experimental 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 and Fridovich 1971).

The CAT activity was determined following the method proposed by Cakmak and Marschner (1992). Briefly, the reaction was initiated following the addition of 25 μl of the crude enzyme extract to 975 μl of a reaction mixture consisting of 50 mM potassium phosphate buffer (pH 6.8) and 20 mM H_2O_2. The determination of CAT activity was based on the rate of 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).

The POX activity was assayed following the colorimetric determination of pyrogallol oxidation according to Kar and Mishra (1976).

The reaction was started after the addition of 15 µl of the crude enzyme extract to 985 µl of a reaction mixture containing 25 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2. The POX activity was measured by the absorbance of colored purpurogallin at 420 nm for 1 min at 25°C. An extinction coefficient of 2.47 mM^{-1} cm^{-1} was used to calculate POX activity (Chance and Maehley 1955).

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

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

The GR activity was assayed following the method described by Carlberg and Mannervik (1985). Briefly, the reaction was initiated 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 that was prepared in 0.5 mM of Tris-HCl buffer (pH 7.5). The decrease in absorbance was determined at 340 nm for 1 min at 30°C. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate GR activity (Foyer and Halliwell 1976).

The methodology proposed by Habig et al. (1974) was used to determine GST activity. First, 75 µl of the crude enzyme extract was added to 925 µl of a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5) and 50 mM reduced GSH. Next, the reaction was initiated 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. The GST activity was determined using an extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig et al. 1974).

The enzyme activity was expressed on a protein basis and its concentration was determined according to the method of Bradford (1976).

Determination of AsA concentration. The AsA concentration was determined following the method described by Kampfenkel et al. (1995). A total of 300 mg of leaf tissue was homogenized in 2 ml of 6% (wt/vol) acid trichloroacetic (TCA) and centrifuged at 15,000 × *g* for 5 min at 4°C. Aliquots of 200 µl of the crude extract 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% (wt/vol) TCA, 800 µl of 42% (vol/vol) H_3PO_4, 800 µl of 4% (wt/vol) 2,2′-dipyridyl (dissolved in 70% ethanol), and 400 µl of 3% (wt/vol) $FeCl_3$ were added to the mixture. After vigorous stirring, the mixture was incubated at 42°C for 40 min. The reaction was then stopped in an ice bath, and the absorbance was measured at 525 nm. The AsA concentration was determined according to an AsA calibration curve (Sigma-Aldrich, São Paulo, Brazil).

Determination of malondialdehyde (MDA) concentration. The oxidative damage in the leaf cells was determined as the concentration of the total 2-thiobarbituric acid (TBA) reactive substances and expressed as MDA equivalents according to Cakmak and Horst (1991). First, 100 mg of leaf tissue was ground into a fine powder using a mortar and pestle while adding liquid nitrogen. Then, the fine powder was homogenized in 2 ml of 0.1% (wt/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 1.5 ml of TBA solution (0.5% in 20% TCA) in a ThermoMixer (Eppendorf, Hamburg, Germany) at 95°C for 30 min. 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 and Packer 1968).

Determination of hydrogen peroxide (H_2O_2) concentration. The method described by Kuo and Kao (2003) was used to assay the concentration of H_2O_2. Initially, a total of 100 mg of homogenized leaf tissue was ground into a fine powder using a mortar and pestle while adding liquid nitrogen. The fine powder was homogenized with an extraction mixture containing 50 mM potassium phosphate buffer (pH 6.5) and 1 mM hydroxylamine in a volume of 2 ml. The homogenate was centrifuged at 10,000 × *g* for 15 min at 4°C and the supernatant was collected. The reaction was initiated with the addition of 100 µl of the supernatant to a 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 2 ml (Gay and Gerbicki 2000). The samples were kept in darkness for 30 min, and 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 for H_2O_2 (Sigma-Aldrich, São Paulo, Brazil).

Determination of superoxide ($O_2^{\cdot -}$) concentration. A total of 200 mg of leaf tissue was ground into a fine powder with a mortar and pestle with added liquid nitrogen to determine the concentration of $O_2^{\cdot -}$. The fine powder was homogenized in an ice bath in 2 ml 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 1.9 ml 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 $O_2^{\cdot -}$ concentration was determined by subtracting the absorbance of the final product from the initial absorbance at 540 nm (Chaitanya and Naithani 1994).

Experimental design and data analysis. An experiment consisting of two cultivars (TMG 132 and Fundacep 59) was arranged in a completely randomized design with 12 replications to evaluate the TSS and the NL. A 2 × 2 × 4 factorial experiment consisting of noninoculated and inoculated plants of two cultivars and four sampling times (4, 6, 8, and 10 dai) with four replications was arranged in a completely randomized design to obtain the leaf samples for the biochemical analysis. All experiments were repeated once. Data from one representative experiment is shown in the Tables and Figures. Each experimental unit consisted of a 2 liter plastic pot with two plants. All data were analyzed using analysis of variance (ANOVA) and the means from the treatments were compared using *t* test ($P \leq 0.05$) using SAS (version 6.12; SAS Institute, Inc., Cary, NC). For TSS, the ANOVA was conducted on a 2 × 4 factorial experiment with two cultivars and four evaluation times (4, 6, 8, and 10 dai). For the NL and AUDPC, a one-way ANOVA was used to analyze the two cultivars. For the biochemical variables, the ANOVA was conducted on a 2 × 2 × 4 factorial design consisting of two cultivars, plant inoculation type (noninoculated and inoculated plants), and four sampling times (4, 6, 8, and 10 dai). The Pearson correlation technique was used to determine the relationships between TSS and the activities of SOD, CAT, POX, APX, GPX, GST, and LOX as well as the concentrations of AsA, MDA, H_2O_2, and $O_2^{\cdot -}$.

RESULTS

NL and AUDPC. The cultivar (C) and sampling time (ST) factors as well as their interaction were significant for TSS, whereas C was significant for NL and AUDPC (Table 1). The pattern of

target spot lesion development on the adaxial surface of the soybean leaves of the TMG 132 and Fundacep 59 cultivars is illustrated in Figure 1A. The leaves of plants from TMG 132 contained numerous, larger necrotic lesions that coalesced and were surrounded by well-developed chlorotic halos in comparison with Fundacep 59. The NL was significantly reduced by 14% in plants from Fundacep 59 compared with plants from TMG 132 (Fig. 1B). Plants from Fundacep 59 showed TSS values that were significantly lower, by 50, 13, 21, and 19% at 4, 6, 8, and 10 dai, respectively, than those of plants from TMG 132 (Fig. 1C). The AUDPC was significantly lower, by 15%, in plants from Fundacep 59 than in plants from TMG 132 (Fig. 1D).

Antioxidant enzymes activities. The factors C, plant inoculation (PI), and ST were significant for SOD, POX, APX, and GR activities as well as for the concentrations of AsA and $O_2^{\cdot-}$ (Table 1). For the activities of CAT and GST, only PI and ST were significant. The factors C and PI were significant for GPX activity and the concentrations of MDA and H_2O_2. The activities of SOD, CAT, POX, APX, GPX, GR, and GST, as well as the concentrations of AsA, MDA, H_2O_2, and $O_2^{\cdot-}$ were significantly influenced by at least one of the two-way and three-way interactions (Table 1). For TMG 132, POX activity significantly increased by 39, 60, 39, and 70%, GPX activity by 63, 77, 82, and 93%, and GST activity by 77, 43, 83, and 44%, respectively, at 4, 6, 8, and 10 dai, for the inoculated plants in comparison with the noninoculated ones (Fig. 2). Significant increases of 51, 76, 70 and 98% for POX activity, of 142, 117, 91, and 127% for GPX activity, of 73, 23, 34 and 50% for GR activity, and of 123, 41, 62, and 85% for GST activity, respectively, at 4, 6, 8 and 10 dai, occurred for the inoculated plants from Fundacep 59 in comparison with the noninoculated ones. For inoculated plants from TMG 132, the activities of SOD, CAT, APX, and GR were significantly greater by 31, 11, and 22% at 6, 8, and 10 dai, by 28% at 6 dai, by 17% at 4 dai, and by 62 and 29% at 4 and 10 dai, respectively, relative to their noninoculated counterparts (Fig. 2). SOD activity significantly increased by 21, 21, and 46% at 6, 8, and 10 dai, CAT by 26 and 15% at 6 and 10 dai, APX by 37, 32, and 68% at 4, 8, and 10 dai, and GR by 73, 23, 34, and 50% at 4, 6, 8, and 10 dai for inoculated plants from Fundacep 59 in comparison with the noninoculated counterparts (Fig. 2). GST activity significantly increased by 9% at 4 dai for the noninoculated plants from TMG 132 in comparison with the noninoculated plants from Fundacep 59 (Fig. 2). For the inoculated plants from Fundacep 59, the activities of SOD, APX, GPX, GR, and GST significantly increased, respectively, by 10 and 19% at 8 and 10 dai, by 19 and 35% at 4 and 10 dai, by 16 and 15% at 4 and 6 dai, by 31 and 18% at 6 and 10 dai, and by 16% at 4 dai, respectively. POX activity significantly decreased by 10% at 4 dai for inoculated plants from TMG 132 in comparison with inoculated plants from Fundacep 59 (Fig. 2).

Concentrations of AsA, MDA, H_2O_2, and $O_2^{\cdot-}$. The factors C, PI, and ST were significant for the concentrations of AsA and $O_2^{\cdot-}$ (Table 1). The factors C and PI were significant for the concentrations of MDA and H_2O_2. The concentrations of AsA, MDA, H_2O_2, and $O_2^{\cdot-}$ were significantly influenced by at least one of the two-way and three-way interactions (Table 1). For TMG 132,

TABLE 1. Analysis of variance of the effects of the cultivar (C), plant inoculation (PI), and sampling time (ST) on target spot severity (TSS), number of lesions (NL) per square centimeter of leaflet, area under the disease progress curve (AUDPC), and the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione S-transferase (GST) as well as the concentrations of ascorbate (AsA), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and superoxide ($O_2^{\cdot-}$) in soybean plants from TMG 132 and Fundacep 59 cultivars inoculated with *Corynespora cassiicola*[a]

Variables	C	PI	ST	C × PI	C × ST	PI × ST	C × PI × ST
TSS	***	–	***	–	***	–	–
NL	***	–	–	–	–	–	–
AUDPC	***	–	–	–	–	–	–
SOD	**	***	***	**	ns	**	ns
CAT	ns	***	**	ns	ns	**	ns
POX	**	***	***	**	ns	**	ns
APX	*	***	***	**	ns	**	ns
GPX	**	***	ns	**	ns	ns	ns
GR	***	***	**	*	ns	***	ns
GST	ns	***	***	ns	ns	***	*
AsA	***	***	***	***	*	ns	ns
MDA	**	***	ns	**	ns	ns	ns
H_2O_2	**	***	ns	**	ns	ns	ns
$O_2^{\cdot-}$	***	***	***	**	ns	***	ns

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

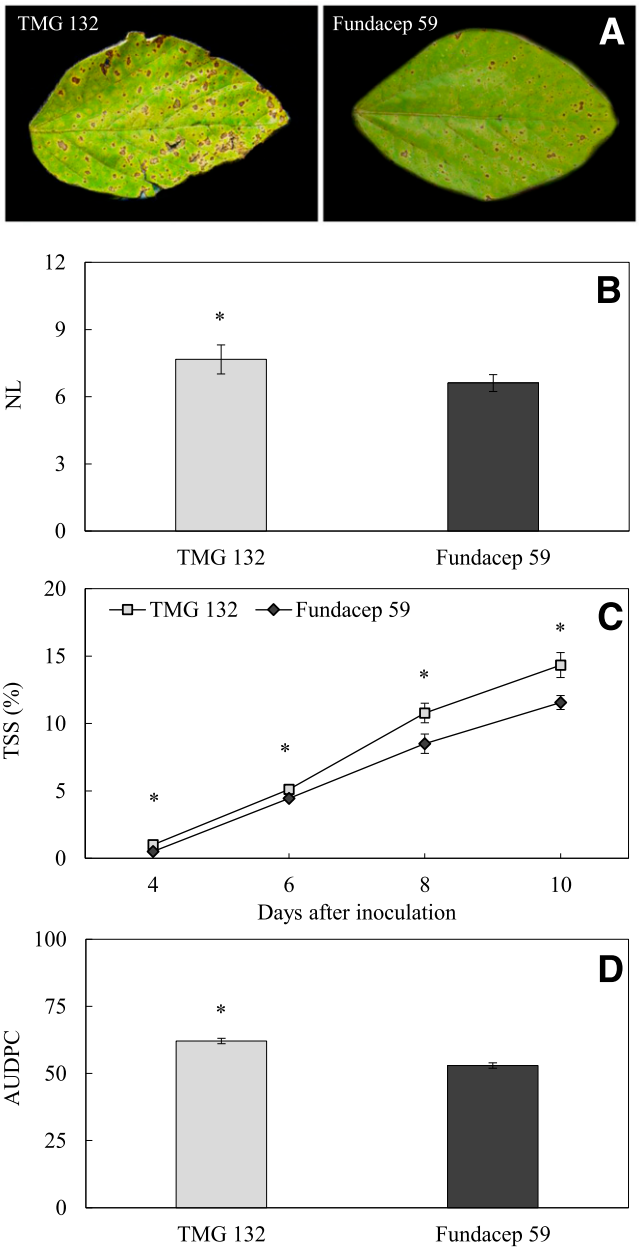

Fig. 1. A, Symptoms of target spot, **B,** number of lesions (NL) per square centimeter of leaflet, **C,** target spot severity (TSS), and **D,** the area under the disease progress curve (AUDPC) on soybean cultivars TMG 132 (susceptible) and Fundacep 59 (resistant) inoculated with *Corynespora cassiicola*. The means marked by an asterisk (*) indicate significant difference ($P \leq 0.05$) as determined using *t* test. The bars represent the standard error of the mean. Two experiments were conducted with similar results, so results from one experiment are shown.

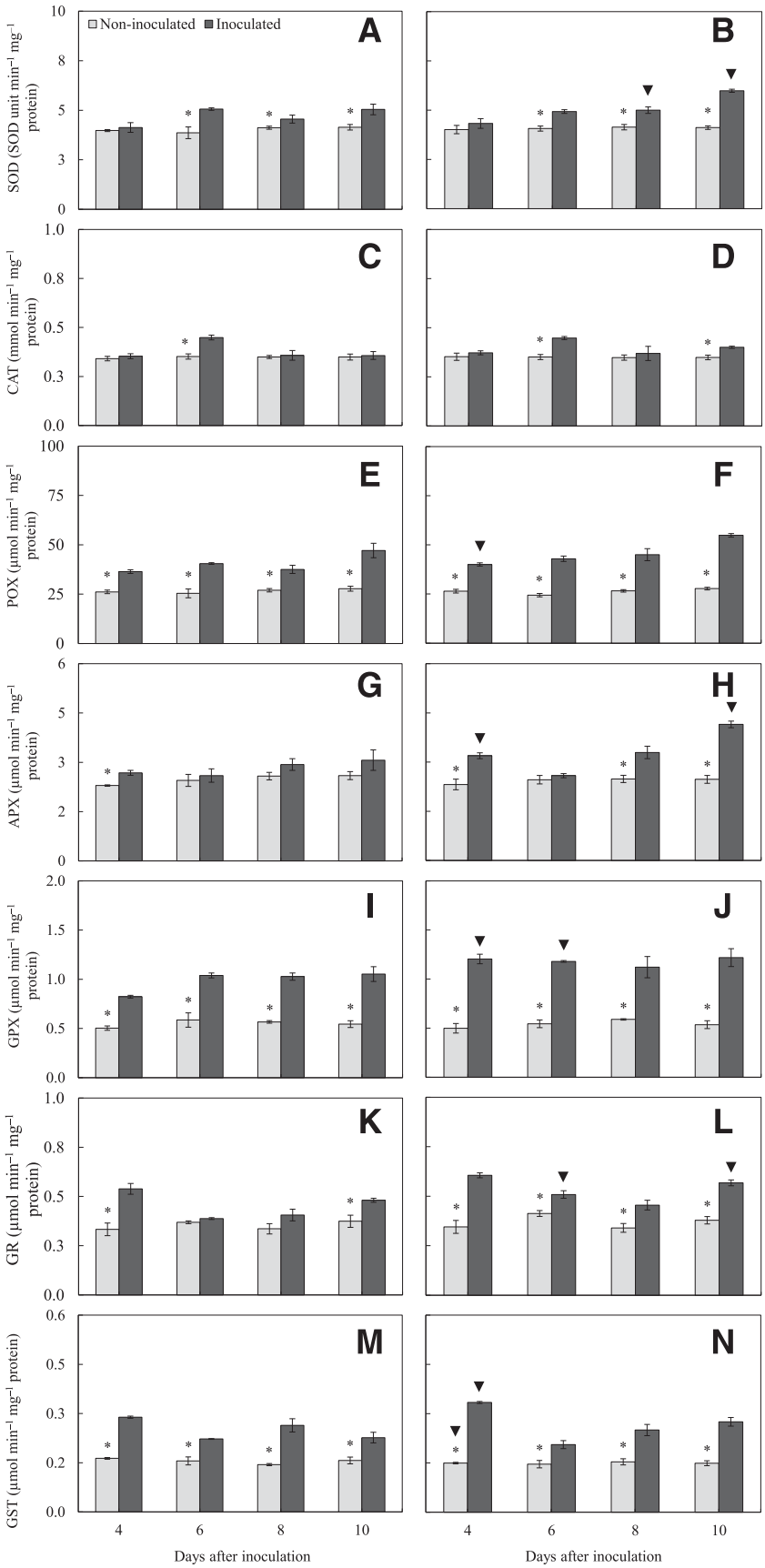

Fig. 2. Activities of **A and B,** superoxide dismutase (SOD), **C and D,** catalase (CAT), **E and F,** peroxidase (POX), **G and H,** ascorbate peroxidase (APX), **I and J,** glutathione peroxidase (GPX), **K and L,** reductase glutathione (GR), and **M and N,** glutathione *S*-transferase (GST) on soybean cultivars TMG 132 (susceptible) (A, C, E, G, I, K, and M) and Fundacep 59 (resistant) (B, D, F, H, J, L, and N) not inoculated (NI) or inoculated (I) with *Corynespora cassiicola*. The means marked by an asterisk (*) indicate significant difference ($P \leq 0.05$) using *t* test between the NI and I treatments. The means marked by an inverted triangle (▼) indicate significant difference ($P \leq 0.05$) using *t* test between cultivars. The bars represent the standard error of the mean. Two experiments were conducted with similar results, so results from one experiment are shown.

AsA concentration significantly increased by 22 and 14% at 6 and 8 dai, MDA concentration by 16, 22, and 36% at 6, 8, and 10 dai, H_2O_2 concentration by 18, 13, 15, and 16% at 4, 6, 8, and 10 dai, and $O_2^{\cdot-}$ concentration by 32, 50, and 85% at 6, 8, and 10 dai, respectively, for the inoculated plants in comparison with the noninoculated ones (Fig. 3). Significant increases of 69, 56, 49, and 52% for AsA concentration at 4, 6, 8, and 10 dai and of 35 and 66% for $O_2^{\cdot-}$ concentration at 8 and 10 dai, respectively, occurred for the inoculated plants from Fundacep 59 in comparison with the noninoculated ones. For inoculated plants from Fundacep 59, AsA concentration significantly increased by 65, 56, 48, and 52%, respectively, at 4, 6, 8, and 10 dai in comparison with plants from TMG 132. The MDA concentration increased by 23% at 10 dai, the H_2O_2 concentration by 11 and 9%, respectively, at 8 and 10, and the $O_2^{\cdot-}$ concentration by 26% at 6 dai for the inoculated plants from TMG 132 in comparison with inoculated plants from Fundacep 59 (Fig. 3).

Correlation analysis. For TMG 132, several significant positive and negative correlations were observed. TSS was positively correlated with the activities of POX, GPX, and $O_2^{\cdot-}$ concentration (Table 2). The SOD activity was positively correlated with both POX activity and AsA concentration and negatively correlated with GST activity. The CAT activity was negatively correlated with the activities of GR and POX and positively correlated with APX activity. The POX activity was positively correlated with AsA concentration and the GST activity was negatively correlated with $O_2^{\cdot-}$ concentration (Table 2). For Fundacep 59, there were positive correlations among TSS, the activities of SOD, POX, and APX and the concentration of $O_2^{\cdot-}$. The POX activity was positively correlated with both APX activity and $O_2^{\cdot-}$ concentration, whereas CAT activity was negatively correlated with GST activity (Table 2).

DISCUSSION

The present study provides evidence that the antioxidant system in soybean plants may play a pivotal role in reducing the ROS damage associated with infection by *C. cassiicola*. The production of ROS in plants due to infection by pathogens is a well-documented phenomenon (Daub et al. 2013; Knörzer et al. 1999). Whatever their origin, ROS accumulation leads to oxidative damage in host cells,

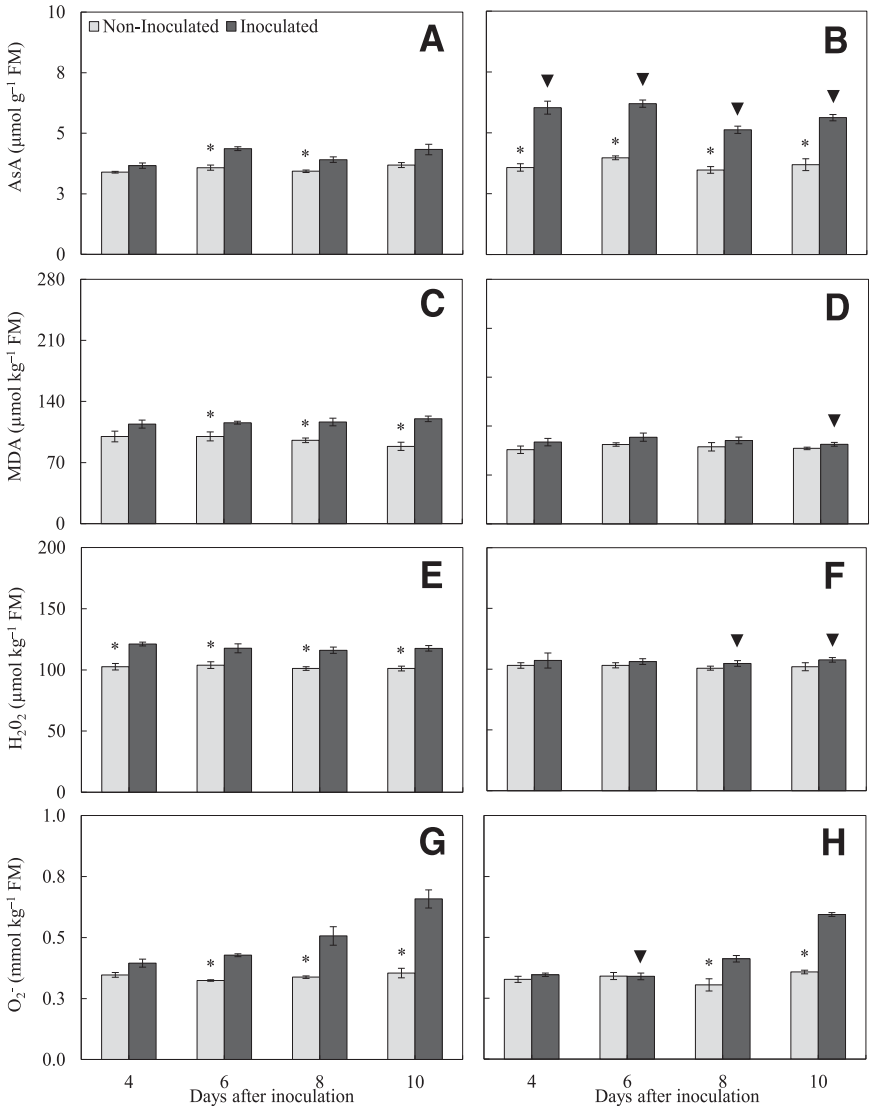

Fig. 3. Concentrations of **A and B,** ascorbate (AsA), **C and D,** malondialdehyde (MDA), **E and F,** hydrogen peroxide (H_2O_2), and **G and H,** superoxide ($O_2^{\cdot-}$) on soybean cultivars TMG 132 (susceptible) (A, C, E, and G) and Fundacep 59 (resistant) (B, D, F, and H) not inoculated (NI) or inoculated (I) with *Corynespora cassiicola*. The means marked by an asterisk (*) indicate significant difference ($P \leq 0.05$) using *t* test between NI and I treatments. The means marked by an inverted triangle (▼) are significantly different ($P \leq 0.05$) using *t* test between cultivars within each sampling time and for the NI or I treatments that are followed. The bars represent the standard error of the mean. Two experiments were conducted with similar results, so the results from one experiment are shown. FM = fresh matter.

which favors infection by necrotrophic pathogens (Daub et al. 2013; Heller and Tudzynski 2011). Consistent with previous studies (Godoy et al. 2012; Pitol et al. 2011), Fundacep 59 were more resistant to target spot than plants from TMG 132 as indicated by the reduced TSS, NL, and AUDPC values. However, information about the underlying mechanisms involved in soybean resistance to target spot is still lacking in the literature.

The production of ROS and lipid peroxidation, considered to be the two biochemical markers for assessing lipid destruction following an oxidative burst, are among the first defense reactions induced at the early stages of fungal infection (Apel and Hirt 2004; Blokhina and Fagerstedt 2010). In the present study, significant increases in $O_2^{\bullet-}$ concentration in response to *C. cassiicola* infection occurred in both TMG 132 and Fundacep 59. However, the inoculated plants from Fundacep 59 were more efficient at the removal of $O_2^{\bullet-}$ in the late stages of *C. cassiicola* infection than TMG 132. These results are in line with the study by Debona et al. (2012) who reported that wheat plants from a resistant cultivar were better able to detoxify $O_2^{\bullet-}$ than those from a susceptible cultivar during the course of *Pyricularia oryzae* infection. The high levels of ROS generated in the roots of tomato plants infected by *F. oxysporum* f. sp. *lycopersici* resulted in an increase in lipid peroxidation and the concurrent production of MDA (Mandal et al. 2008). Similarly, an increase in MDA concentration was observed for the inoculated plants from TMG 132 in this study. Because MDA is a product of lipid peroxidation, the lower concentrations of $O_2^{\bullet-}$ recorded for plants from Fundacep 59 could explain, at least in part, their lower MDA concentration than those of the plants from TMG 132.

The SOD is one of the most important scavenger enzymes and represents the first line of defense against ROS through its catalysis of the dismutation of $O_2^{\bullet-}$ to H_2O_2 and oxygen (O_2) (Giannopolitis and Ries 1977; Gill and Tuteja 2010; Hao et al. 2011). In the present study, SOD activity increased for plants from both cultivars in response to *C. cassiicola* infection, which is consistent with what has been obtained for the tomato–*B. cinerea* (Kużniak and Skłodowska 2005), strawberry–*Mycosphaerella fragariae* (Ehsani-Moghaddam et al. 2006), and wheat–*P. oryzae* (Debona et al. 2012) interactions. However, in this study, a high degree of SOD activity was recorded in the late stages of *C. cassiicola* infection for plants from Fundacep 59 compared with plants from TMG 132. Therefore, the SOD activity contributed to the lower $O_2^{\bullet-}$ concentration and the reduced damage to the plant cell plasma membrane observed in the former cultivar as indicated by the lower concentration of MDA. In strawberry and wheat plants infected with *Mycosphaerella fragariae* (Ehsani-Moghaddam et al. 2006) and *P. oryzae* (Debona et al. 2012), respectively, greater concentrations of SOD activity in plants from resistant cultivars than susceptible ones were observed in response to fungal infection. Therefore, it is believed that SOD, by reducing the $O_2^{\bullet-}$ concentration and the consequent cellular damage, plays an important role in soybean resistance to target spot.

The H_2O_2 is another ROS generated during pathogen infection or by $O_2^{\bullet-}$ dismutation via SOD (Lanubile et al. 2012). For this reason, an increase in the activities of CAT, APX, and POX, which are involved in H_2O_2 removal, is required to lower the concentration of H_2O_2 (Foyer and Noctor 2011; Gill and Tuteja 2010; Mittler et al. 2004). The findings of this study highlighted that the activities of CAT, APX, and POX increased upon *C. cassiicola* infection. The activity of peroxisomal CAT on tomato plants in response to *B. cinerea* infection was greater but was followed by a strong decline during the late stages of fungal infection compared with noninoculated plants (Kużniak and Skłodowska 2005). In the current study, the activities of CAT, APX, and POX were greater in plants from Fundacep 59 than plants from TMG 132. In agreement with these results, Magbanua et al. (2007) demonstrated that CAT activity was greater in the resistant maize lines to *Aspergillus flavus* than in susceptible ones. In the wheat–*Rhizoctonia cerealis* interaction, POX activity was greater in a resistant than in a susceptible cultivar (Hong-xia et al. 2011). The high POX and APX activities were also associated with wheat resistance to leaf blast (Debona et al. 2012). Polkowska-Kowalczyk et al. (2007) demonstrated that in the *Solanum tuberosum–Phytophthora infestans* interaction, APX activity was significantly greater in the resistant cultivar than in the susceptible one. The increases in the activities of CAT, POX, and APX in the inoculated Fundacep 59 plants could have protected the plant cells from the cytotoxic effects of H_2O_2.

The AsA is considered to be one of the most powerful ROS scavengers and acts as an electron donor to APX to reduce H_2O_2 to water (Foyer and Noctor 2011). In the present study, the AsA concentration in plants from both cultivars increased upon *C. cassiicola* infection. However, a greater accumulation of AsA was observed in plants from Fundacep 59 than in plants from TMG 132. In the *S. tuberosum–P. infestans* interaction, the concentration of AsA, as well as the activity of APX were also significantly greater in the resistant than in the susceptible cultivar. Furthermore, it has been suggested that APX and AsA played an important role in the regulation of ROS levels upon *P. infestans* infection (Polkowska-Kowalczyk et al. 2007). These findings also support what was proposed for the soybean–*C. cassiicola* interaction in the present study.

The GPX and GST are important enzymes for the alleviation of oxidative stress in plant cells and they use GSH to reduce H_2O_2 and other hydroperoxides (Gill and Tuteja 2010). In the inoculated plants from TMG 132 and Fundacep 59, there was a consistent increase in the activities of GPX and GST during the *C. cassiicola* infection process. In contrast, Debona et al. (2012) observed

TABLE 2. Pearson correlation coefficients among target spot severity (TSS), the activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione S-transferase (GST) and the concentrations of ascorbate (AsA), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and superoxide ($O_2^{\bullet-}$) in soybean plants from TMG 132 (above the diagonal) and Fundacep 59 (below the diagonal) cultivars inoculated with *Corynespora cassiicola*[a]

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

[a] Levels of probability: [ns] = not significant, * = 0.05, ** = 0.01, and *** = <0.0001.

increases in GPX activity only for the inoculated plants from a susceptible cultivar in the wheat–*P. oryzae* interaction. The activities of GPX and GST were greater in plants from Fundacep 59 than in plants from TMG 132 in the early stages of *C. cassiicola* infection. Accordingly, GST appeared to be one of the most important antioxidant enzymes involved in wheat resistance to leaf blast (Debona et al. 2012). In contrast, Polkowska-Kowalczyk et al. (2007) demonstrated that a resistant genotype of potato showed lower GST activity in response to *P. infestans* infection. Therefore, it seems reasonable to assume that GPX and GST play a key role in soybean resistance to target spot through the removal of H_2O_2 and, therefore, the products contributing to lipid peroxidation.

The GR belongs to the Foyer-Halliwell-Asada pathway, whose function is to scavenge ROS in the plastids and, possibly, the extraplastidic compartments of the plant cell (Foyer and Noctor 2011; Noctor and Foyer 1998). The activity of GR was found to be greater in the inoculated plants than in the noninoculated ones, especially for Fundacep 59. Conversely, at the early stages of *B. cinerea* infection in tomato plants, GR activity decreased in comparison with noninfected plants (Kużniak and Skłodowska 2005). The level of GR activity in plants from a resistant apricot cultivar was greater than in a susceptible one following infection with *Plum pox virus* (Hernández et al. 2001). In a study involving potato and an elicitor derived from *P. infestans*, the increase in GR activity at an early phase following treatment with a culture filtrate was greater in the susceptible than in the resistant cultivars (Polkowska-Kowalczyk et al. 2007). However, the data from the present study suggest that higher GR activity in the plants from both cultivars, but to a greater extent in the resistant one, contribute to the maintenance of high levels of GSH and the alleviation of the oxidative stress caused by *C. cassiicola* infection.

The results from the present study indicate that a more efficient antioxidative system, which is involved in the removal of ROS generated in soybean plants during *C. cassiicola* infection, reduces cellular damage and contributes to increased resistance to target spot. Further research that aims to investigate the antioxidant 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 subject to severe epidemics of target spot.

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