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Research Article

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Role of Antioxidant Enzymes, Hydrogen Peroxide and PR-Proteins in the Compatible and Incompatible Interactions of Cowpea (*Vigna unguiculata*) Genotypes with the Fungus *Colletotrichum gloeosporioides*

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Abstract

Several diseases, such as anthracnose, which are caused by the fungus species *Colletotrichum gloeosporioides*, negatively affect the cultivation of the cowpea (*Vigna unguiculata*). This work was conducted to measured the time-course activities and evaluate the possible roles of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and hydrogen peroxide (H_2O_2) and the pathogenesis-related proteins (PR-proteins), peroxidase (POX), β-1,3-glucanase (GLU) and chitinase (CHI), in the resistant (TE97) and susceptible (BR3) cowpea genotypes in response to *C. gloeosporioides* infection. During infection, the SOD activity was increased, while the CAT activity was decreased in TE97. The APX activity in TE97 was similar to that in BR3 at 12 to 24 after infection (HAI). These data were consistent with the increased production and accumulation of leaf H_2O_2 within this time period in TE97; the generation of H_2O_2 was also observed in BR3, but to a lesser extent. The fungal inoculation also induced changes in the PR-proteins analyzed. Overall POX, GLU and CHI activities were higher in the resistant genotype TE97 compared with those in the susceptible genotype BR3. Altogether, these results showed that H_2O_2 and the PR-proteins play important roles in the interactions of cowpea and *C. gloeosporioides*.

Keywords

Cowpea; *Colletotrichum gloeosporioides*; Antioxidant enzymes; PR-proteins; Plant defense

Abbreviations

APX: Ascorbate Peroxidase; βGLU: β-1,3-glucanase; CAT: Catalase; CHI: Chitinase; DAB: 3′,3′-diaminobenzidine; diaminobenzidine; HAI: Hours After Inoculation; HR: Hypersensitive Response; NBT: Nitroblue Tetrazolium, PCD: Programmed Cell Death; POX: Peroxidase; SOD: Superoxide Dismutase; TCA: Trichloroacetic Acid

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Introduction

After pathogen recognition, highly localized biochemical events are rapidly induced to inhibit the further development of the attacking pathogen. The rapid production and accumulation of reactive oxygen species (ROS), particularly the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) [1], culminates with a hypersensitive response (HR) and localized programmed cell death (PCD) to impair the pathogen establishment and development inside the host tissues and deprive the pathogen of further access to nutrients. Concomitant with or following HR, several pathogen defense-related genes are translated into antioxidant enzymes and PR-proteins [2] that confer resistance to the plant in defense against pathogen attack.

Plants possess a number of antioxidant enzymes that eliminate ROS. Superoxide dismutase (SOD) catalyzes the conversion of the superoxide free radical (O_2^-) to molecular oxygen and H_2O_2 [3]. The production of SOD is specifically induced during oxidative stress, when the levels of superoxide in the cell exceed the spontaneous dismutation rate. H_2O_2 is eliminated by catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) and other scavenging enzymes, such as glutathione peroxidase (GPX) and various peroxiredoxins (Prx) [1], leading to oxidative stress tolerance and pathogen resistance. Beside to ROS generation and scavenging, HR, PCD and reinforcement of the cell wall, plants respond to pathogen attack using a variety of compounds and proteins. Pathogenesis-related proteins (PR-proteins) are specifically induced in response to infection by pathogens such as fungi, bacteria, and viruses, or to adverse environmental factors [2]. Presently there are 17 recognized families of PR-proteins, including β-1,3-glucanase (PR-2 family), chitinase type I, II, IV, V, VI, VII (PR-3), chitinase type I, II (PR-4), chitinase type III (PR-8), chitinase type I (PR-11) and peroxidase (PR-11) [2]. Peroxidases (POXs) are enzymes that catalyze the oxidation of several substrates at the expense of H_2O_2, playing a key role in the detoxification of H_2O_2 during the H_2O_2-dependent polymerization of hydroxycinnamyl alcohols involved in the lignification process (lignin biosynthesis) and H_2O_2-dependent cross-linking of cell wall proteins, such as hydroxyproline-rich glycoproteins and proline-rich proteins associated with the reinforcement of plant cell walls [4]. Several studies have shown POX induction associated with the defense against viruses [5], bacteria [6], fungi [7], nematodes [8], and insects [9]. Khairullin et al. [10] showed the association of an anionic wheat peroxidase with chitin, and recently, Maksimov et al. [11] reported a similar association for both cationic and anionic isoforms from various plants species, which suggested the participation of POXs in plant defense mechanisms against chitin-containing fungi. Indeed, it has been reported that some POXs possess antifungal activity [12,13].

Cell wall appositions, such as papillae, also represent an important barrier to pathogen penetration. In cereal-powdery mildew interactions, papillae are formed on the inner side of the outer epidermal cell wall subjacent to primary and appressorial germ tubes in response to pathogen attack. Many compounds accumulate in these papillae, such as callose, proteins, and phenolic and guanidine compounds [14,15].

The fungal cell wall is primarily composed of glucans, chitin,

mannans and glycoproteins. Glucans are major structural polysaccharides that constitute approximately 50–60% of the fungal wall dry weight. An estimated 65-90% of the cell wall glucans are β-1,3-glucans. Chitin is composed of β-1,4-linked N-acetylglucosamine residues and is typically less abundant than either the glycoprotein or glucan fractions, as it represents only 10-20% of the cell wall of filamentous fungi. Glucans bind to chitin to form the chitin-glucan complex (CGC). The CGC is present in all fungi, except zygomycetes, and cross-links with glycoproteins to form the structural basis of the fungal cell wall, providing mechanical strength and integrity [16].

β-1-3-glucanases (GLUs) are enzymes that predominantly promote the endohydrolytic cleavage of β-1,3-glucosidic linkages in β-1,3-glucans. Plant GLUs are involved is several physiological functions and developmental processes, such as cell division, microsporogenesis, pollen germination, tube growth, fertilization, embryogenesis, fruit ripening, seed germination, mobilization of storage reserves, and bud dormancy. In addition, these enzymes have been implicated in the response to pathogen attack, wounding, cold, ozone and UV-B [17].

Chitinases (CHIs) act on the β-1,4-glycosidic linkage of chitin. In plants, CHIs have also been implicated in the mechanisms of resistance against pathogens and insect pests, and other functions, such as nodulation, embryogenesis, and functions unrelated to their catalytic activity, including antifreeze proteins and inhibitors of α-amylases [18]. Several CHIs inhibit the growth of many fungi through the lysis and disarrangement of the cell wall structure, particularly in combination with GLUs. The combined expression of CHI and GLU genes in rice (*Oryza sativa* L. subsp. *indica* variety Pusa Basmati 1) enhanced resistance against *Rhizoctonia solani* [19]. Moreover, at the cell wall, CHIs can release chitin oligomers, namely pathogen (microbe)-associated molecular patterns (PAMPs or MAMPs, respectively) [20], which activate a variety of plant defense responses, including ROS generation, the production and export of anti-microbial compounds and the fortification of the plant cell wall [21].

The cowpea is one of the most important legumes for human consumption, particularly in tropical and subtropical regions of Africa, Asia and South America [22]. The production of this crop is negatively affected by several diseases, including anthracnose, caused by fungal species of the *Colletotrichum* genera, which are among the most potent plant pathogenic fungi and cause severe losses worldwide [23].

The objective of this present work was to evaluate the possible role of the antioxidant enzymes (SOD, CAT, and APX), H_2O_2, and the PR-proteins (POX, GLU, and CHI) in the infection of two cowpea genotypes, resistant (TE97) and susceptible (BR3) [24], with the hemibiotrophic fungus *Colletotrichum gloeosporioides*.

Materials and Methods

Biological materials and inoculation

The isolate of the hemibiotrophic fungus *C. gloeosporioides* [(Sacc. & Magnus) Briosi & Cav.] was previously identified [24] and cultured on potato dextrose agar (PDA, Difco, Detroit, MI) under continuous fluorescent light at 25°C. The spore suspensions were prepared by washing the surface of 12-d-old cultures with sterile distilled water and passing the suspension through a four-layer muslin cloth to remove fungal mycelia and other debris. The conidium suspension was adjusted in sterile water to a known concentration after counting in a Neubauer chamber under a microscope (Olympus System BX60) and used as inoculum.

The seeds of the resistant (TE 97-411-1E [hereafter, TE97]) and susceptible (BR 3 Tracuateua [hereafter, BR3]) cowpea were obtained from Embrapa Meio-Norte (Piauí, Brazil). The seeds were surface disinfected with 1% (v/v) hypochlorite (0.05% active chloride) for 3 min, rinsed exhaustively with distilled water, soaked in distilled water for 10 min, and sown in 0.5-L pots containing autoclaved (120 °C, 1.5 KGF, 30 min) river sand. The seeds were cultivated at 27-35°C in a greenhouse exposed to 12 h natural light gradients varying from 300-650 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), at canopy level, and daily irrigated with autoclaved (120 °C, 1.5 KGF, 20 min) water for up to 4 days after sowing. Subsequently, the seedlings were irrigated with a 5-fold diluted nutritive solution [25]. Ten days after sowing, visually healthy plantlets were selected and transferred to a growth chamber at 25-30°C, 85 ± 5% relative humidity with a 12 h photoperiod at an intensity of approximately 280 μmol s^{-1} m^{-2} photosynthetically active radiation (PAR). Two days later, the primary leaves were inoculated by applying two 25 μL equidistant droplets of *C. gloeosporioides* spore suspension (4.0 x 10^5 mL^{-1} in sterile distilled water) at each side of the adaxial leaf blade separated by the main vein. The control plants were inoculated with sterile water. The primary leaves (2 per plant) of six individual plants (n = 12) were excised at 0, 12, 24, 48, 72, and 96 hours after inoculation (HAI) and used for subsequent analyses. The experiment was repeated three times.

Hydrogen peroxide (H_2O_2) accumulation

To visualize H_2O_2 accumulation in the cowpea primary leaves using light microscopy, DAB (3´-3´-diaminobenzidine; Sigma) was infiltrated according to Thordal-Christensen et al. [14]. Briefly, the stems of cowpea plantlets collected at six different periods after inoculation with *C. gloeosporioides*, as previously described, were cut at 2 cm above the cotyledon insertion region, and the cut end of the upper plantlet was immersed in a solution containing 1.0 mg DAB mL^{-1}. DAB was dissolved in Milli-Q grade water adjusted initially to pH 3.0 with 1.0 N HCl and heated to 50 °C, followed by the addition of 1.0 N NaOH, pH 4.0. After an 8-h treatment, the cowpea leaves were decolorized by incubation in 1.5 g L^{-1} TCA in a 3:1 (v/v) mixture of ethanol + chloroform for 48 h with at least three changes of the bleaching solution and examined by light microscopy. To detect papilla (callose) formation, the leaf pieces were soaked with phosphate buffer (0.1 M KH_2PO_4/K_2HPO_4, pH 9.0) for 24 h, and the leaf pieces with infection sites were placed on glass slides in phosphate buffer, as previously described [24]. Subsequently, the samples were stained with a 0.1 g L^{-1} buffered solution of aniline blue for 2 h [26]. The leaf pieces were mounted on a microscope glass slide, cleared by drop washing with concentrated HCl (ca. 2 min), and covered with a glass cover slip in glycerol [27]. To visualize the fungal structures, the DAB-treated leaf pieces were further stained with 0.5 g L^{-1} aniline blue in lactophenol for 2-3 min at 70 °C [28]. The fungal structures were strongly stained blue. Light microscopy was performed using an Olympus System Model BX60F5 microscope (Olympus Optical Co. Ltd, Japan). Images were acquired using an Olympus photomicrography system PM-20. The experiments were repeated three times with three replications.

The quantitative production of H_2O_2 was detected using spectrometry, as previously reported [29]. The primary leaves

of control and infected BR3 and TE 97 cowpea genotypes were collected at six different time periods after treatment. The leaves were homogenized (1:5, m/v) in 50 mM borax-borate extraction buffer (0.61 g of boric acid in 150 mL of Milli-Q grade water and 0.95 g of sodium tetraborate in 50 mL of Milli-Q grade water, adjusted to pH 8.4) using a mortar and pestle. The homogenate was filtered through one layer of cheesecloth and centrifuged at 12,000 ×*g* for 20 min at 4°C. The supernatant was used in the assay reaction mixture, which consisted of 0.2 mL of the supernatant + 1.0 mL Solution A + 10 mL solution B. Solution A contained 25 mM $FeSO_4$ + 25 mM $(NH_4)_2SO_4$ + 25 mM H_2SO_4. Solution B consisted of 0.125 mM xylenol orange + 100 mM sorbitol. The H_2O_2 accumulation was calculated based on the standard curve generated using freshly prepared H_2O_2 solutions of known concentrations (0-8.0 nmol H_2O_2/1.2 mL). H_2O_2 concentration was expressed as nmol H_2O_2 per gram leaf fresh weight (nmol H_2O_2 g^{-1} FW)

Enzyme extraction and activity assays

The primary leaves of the TE97 and BR3 genotypes which were inoculated and mock-uninoculated (control) with *C. gloeosporioides* were separately homogenized on ice in extraction buffer (0.5 M Na-acetate buffer, pH 5.2, containing 0.5 M NaCl) using a mortar and pestle. The homogenates were filtrated through one layer of cheesecloth and centrifuged at 10,000 ×*g* for 20 min at 4°C. The supernatants were dialyzed exhaustively against the extraction buffer and used as sources of antioxidant enzymes and PR-proteins.

Superoxide dismutase (SOD; EC 1.15.1.1) activity assay was based on a previously described method [30], which measures the inhibition of the photochemical reduction of NBT (Nitroblue-tetrazolium; Sigma), induced by this enzyme. Different volumes (0.050, 0.060, 0.070, 0.080 and 0.090 mL) of the dialyzed leaf extracts were added into the wells of a microtiter plate containing 0.02 mL of 500 mM sodium phosphate buffer, pH 7.8, 0.01 mL of 0.50% Triton X-100, 0.02 mL of 130 mM L-methionine, 0.01 mL of 2.0 mM EDTA, 0.02 mL of 0.75 mM NBT, 0.02 mL of 1.0 mM riboflavin and Milli-Q grade H_2O to 0.20 mL final volume. The reaction took place in a chamber under illumination by a 32-W fluorescent lamp and was terminated after 5 min by turning off the illumination. The blue formazan produced by NBT photoreduction was measured as the increase in absorbance at 630 nm (Automated Microplate Reader, model ELX800-Bio-Tek Instruments®, Inc., USA). The control reaction mixture was prepared in the same manner as the experimental reaction but without the enzyme extract and maintained in the dark. SOD activity was calculated as the difference between the absorbance of control and that of its experimental equivalent estimated for a one-min reaction. One unit of SOD activity (1 UA) was defined as the amount of sample required to inhibit 50% of the NBT photoreduction [31]. The enzyme activity was expressed in units of activity per gram of leaf fresh weight (UA g^{-1} FW).

Catalase (CAT; EC. 1.11.1.6) activity was assayed as previously described [32]. The dialyzed leaf extract (0.05 mL) was added to 0.95 mL of 12.5 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0. The reaction mixture was incubated at 30 °C. The CAT activity was measured by the decrease in absorbance at 240 nm, in 30-second intervals up to 2 min [33]. CAT activity was calculated using the molar extinction coefficient of 36 x 10^3 mM^{-1} cm^{-1} [34] and expressed as µmol H_2O_2 oxidized per gram of leaf fresh weight per min (µmol H_2O_2 g^{-1} FW min^{-1}).

Ascorbate peroxidase (APX; EC. 1.11.1.11) was determined according to the methodology of Koshiba [35]. The reaction mixture consisted of 0.83 mL of 50 mM potassium phosphate buffer, pH 6.0, containing 0.5 mM ascorbate, 0.10 mL of 2.0 mM H_2O_2 and 0.07 mL of the dialyzed leaf extract. Total APX activity was measured by monitoring the decrease in absorbance at 290 nm for 3 min as a measure of ascorbate oxidation. The APX activity was calculated on the basis of a standard curve built using known concentrations of ascorbate (0.1-1.0 µmol ascorbate mL^{-1}). The APX activity was expressed as µmol oxidized ascorbate (Asc) per gram of leaf fresh weight per min (nmol Asc g^{-1} FW min^{-1}).

Guaiacol peroxidase (POX; EC 1.11.1.7) was measured according to the method of Urbanek et al. [36], using guaiacol as substrate and H_2O_2 as a co-substrate. Aliquots of 0.02 mL of the dialyzed leaf extract, previously diluted (6X) were mixed with 0.98 mL of 50 mM sodium acetate buffer, pH 5.2, 0.5 mL of 60 mM H_2O_2 and 0.5 mL of 20 mM guaiacol in a total volume of 2.0 mL. The reaction mixture was incubated at 30 °C for 10 min, and the absorbance was measured at 480 nm. The variation of one unit of absorbance per min was defined as one unit of peroxidase activity (1 UA) and expressed per gram of leaf fresh weight (UA g^{-1} FW).

β-1,3-glucanase (GLU, EC 3.2.1.39) was assayed based on the amount of glucose liberated by the action of the enzyme on laminarin (Sigma Chemical Company) used as substrate [37]. The amount of glucose liberated was calculated using a standard curve created with known amounts (7.5-240 µg mL^{-1}) of the sugar. The activity was expressed as nanokatal per gram fresh leaf weight (nkat g^{-1} FW). One nkat was defined as 1.0 nmol of D-glucose liberated from laminarin per second under the assay conditions.

Chitinase (CHI, EC 3.2.1.14) assay was conducted colorimetrically [37] by measuring the amount of N-acetyl-D-glucosamine (NAG) produced [38] by the combined hydrolytic action of chitinases and β-glucuronidase on non-radioactive colloidal chitin [39] used as substrate. The amount of NAG produced was calculated using a standard curve produced with known concentrations (100-700 ng mL^{-1}) of commercial NAG dissolved in 0.050 M sodium acetate buffer (pH 5.2) The assay was performed five times, and the CHI activity was expressed as nanokatal per gram fresh leaf weight (nkat g^{-1} FW). One nkat was defined as 1.0 nmol of NAG produced per second at 37 °C.

Statistical analyses

Data from the enzyme assays and quantitative production of H_2O_2 were subjected to analysis of variance (ANOVA) followed by Tukey's test.

Results

Kinetics of the enzymes involved in the oxidative stress

The basal level of SOD (Figure 1A) in the primary leaves of the cowpea was measured at the beginning of the experimental period (0 HAI), and a slight but significantly ($\rho \leq 0.05$) higher level of expression was detected in TE97 (111.93 ± 1.06 AU g^{-1} FW) compared with BR3 (96.33 ± 0.37 AU g^{-1} FW). The results of the time-course experiment showed that the activity of SOD was induced in both cowpea genotypes upon *C. gloeosporioides* infection. However, for the resistant genotype TE97, increased activity was observed at 12 to 24 hours after inoculation (HAI), when the activity (230.53 ± 4.64 AU g^{-1} FW) was significantly ($\rho \leq 0.05$) higher (1.7-fold) compared with

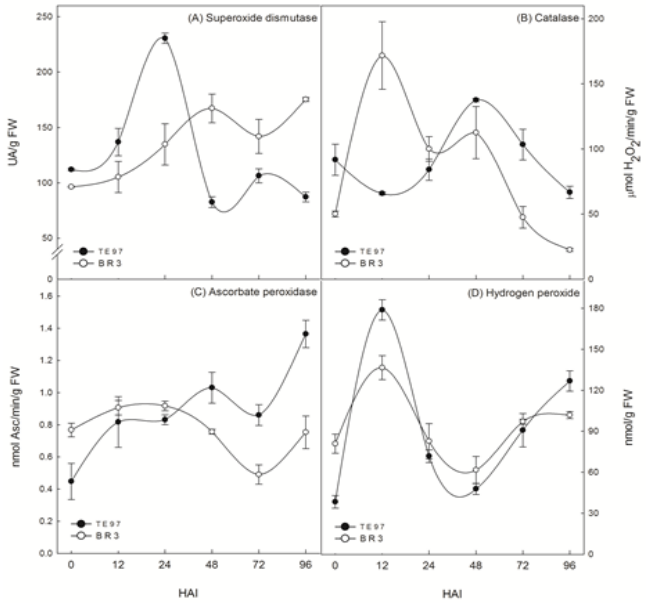

Figure 1: Kinetics of the antioxidant enzymes SOD (A), CAT (B), and APX (C), and H_2O_2 (D) in the leaves of *C. gloeosporioides*-infected cowpea (*Vigna unguiculata*), genotypes TE97 (resistant, -●-) and BR3 (susceptible, -o-). Each data point represents the mean of three independent experiments ± standard error (bar).

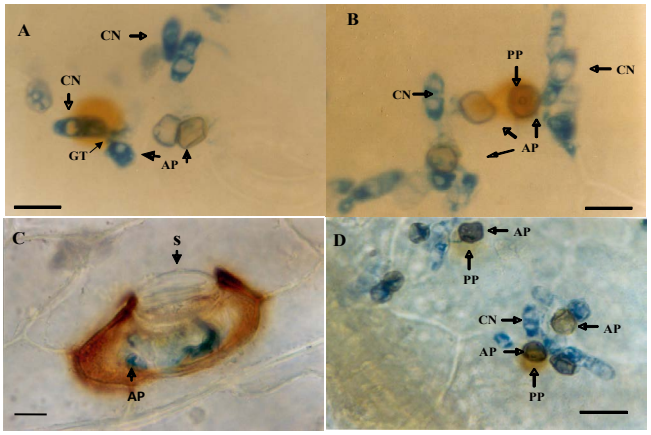

Figure 2: (A) H_2O_2 accumulation around conidium (CN) and germ tube (GT) of *C. gloeosporioides* in the primary leaves of the resistant cowpea (*V. unguiculata*) genotype TE97 at 72 hours after inoculation (HAI), (B) H_2O_2 accumulation in the papilla (PP) region (dark yellow halo), around the appressoria (AP) of *C. gloeosporioides* in the primary leaves of TE97 at 72 HAI. (C) Cell presenting HR with H_2O_2 accumulation in the primary leaves of TE97 at 48 HAI. (D) H_2O_2 accumulation in the papilla (PP) region (dark yellow halo), around the appressoria (AP) of *C. gloeosporioides* in the primary leaves of the susceptible cowpea genotype BR3 at 48 HAI. S = stoma. The leaves were stained with diaminobenzidine (DAB) and lactophenol aniline blue. Bar = 10 µm.

that of the susceptible genotype BR3 (134.71 ± 18.64 AU g^{-1} FW). Thereafter (48 to 96 HAI), contrasting SOD kinetics were observed, as the BR3 genotype presented increasing activities, whereas TE97 experienced a drastic decline, and the activity remained below basal level (0 HAI) until the end of the experimental period.

The basal level (0 HAI) of CAT in the dialyzed extracts of cowpea primary leaves was higher for TE97 (91.67 ± 12.02 µmol H_2O_2 g^{-1} FW min^{-1}) than for BR3 (50.00 ± 2.36 µmol H_2O_2 g^{-1} FW min^{-1}) (Figure 1B). However, at 12 HAI with *C. gloeosporioides*, the CAT activity of TE97 decreased to 65.83 ± 1.18 µmol H_2O_2 g^{-1} FW min^{-1}, whereas for BR3, this activity increased to 171.67 ± 25.93 µmol H_2O_2 g^{-1} FW min^{-1}, which was significantly (ρ≤0.05) higher than that of TE97. Thereafter, from 48-96 HAI, the CAT activity in BR3 decreased, whereas the activity was significantly (ρ≤0.05) increased in TE97.

The results of the time-course experiment showed that the overall activity of APX in the primary leaves of TE97 gradually increased from 0 to 96 HAI (Figure 1C). Accordingly, the basal value for TE97 at 0 HAI was 0.45 ± 0.11 µmol Asc. g^{-1} FW min^{-1}, the APX activity doubled at 12 HAI (0.82 ± 0.16 µmol Asc. g^{-1} FW min^{-1}) and was three times higher after 96 HAI (1.36 ± 0.08 ηmol Asc. g^{-1} FW min^{-1}). In BR3, a slight but not significant increase in APX activity was observed at 0 to 24 HAI, which subsequently decreased at 24 to 72 HAI and returned to basal levels at 96 HAI. Thus, the APX activity of TE97 increased to levels that were significantly (ρ≤0.05) higher than those of BR3 from 48 to 96 HAI.

H_2O_2 accumulation

The H_2O_2 kinetic patterns from 0 to 96 HAI were biphasic for both genotypes, but the patterns differed with regard to accumulation in primary leaves inoculated with *C. gloeosporioides* (Figure 1D). The H_2O_2 level in the resistant genotype TE97 at 12 HAI was (178.77 ± 7.39 nmoles g^{-1} FW) 4.7 times more elevated compared with the basal level (38.36 ± 4.62 nmoles g^{-1} FW), whereas, at this same time point, the H_2O_2 accumulation in BR3 (136.53 ± 8.81 ηmoles g^{-1} FW) showed an increase of only 1.69-fold compared with the basal concentration of H_2O_2 (80.90 ± 6.96 nmoles g^{-1} FW). Thus, at 12 HAI, the H_2O_2 accumulation in TE97 was 31% higher than in BR3. Thereafter, the H_2O_2 concentrations in both genotypes gradually decreased up to 48 HAI. Subsequently, both genotypes showed increased H_2O_2 accumulation, but at 96 HAI, the accumulation of H_2O_2 in TE97 (126.74 ± 7.42 nmoles g^{-1} FW) was significantly higher than that in BR3 (101.83 ± 2.55 nmoles g^{-1} FW).

Macroscopic analysis

The visual examination of cowpea leaves inoculated with *C. gloeosporioides* revealed the presence of small necrotic lesions in the resistant genotype (TE97), while in BR3, well-developed lesions that enlarged over the experimental period were observed, consistent with the observations obtained in our previous study [24].

H_2O_2 generation during papillae development and HR

In response to *C. gloeosporioides* infection, cowpea leaves form papillae (PP, brownish halo) beneath the appressoria (AP) in both genotypes (Figures 2A and 2B). In addition, as from 48 h to 96 h the H_2O_2 levels increased again over that of the basal level (0 h) for both the *C. gloeosporioides*-resistant cowpea genotype TE97 and the susceptible BR3 genotype (Figure 1D), photomicrographs taken at 72 HAI showed that H_2O_2 accumulated at the sites of penetration, beneath and/or radial to the melanized fungus appressoria and papillae (Figures 2A-2C) in both cowpea genotypes, and that TE97 epidermal cells undergone HR (Figure 2D), which were reddish-brown in color [23].

Kinetics of PR-proteins

A gradual and significant (ρ≤0.05) increase of POX activity

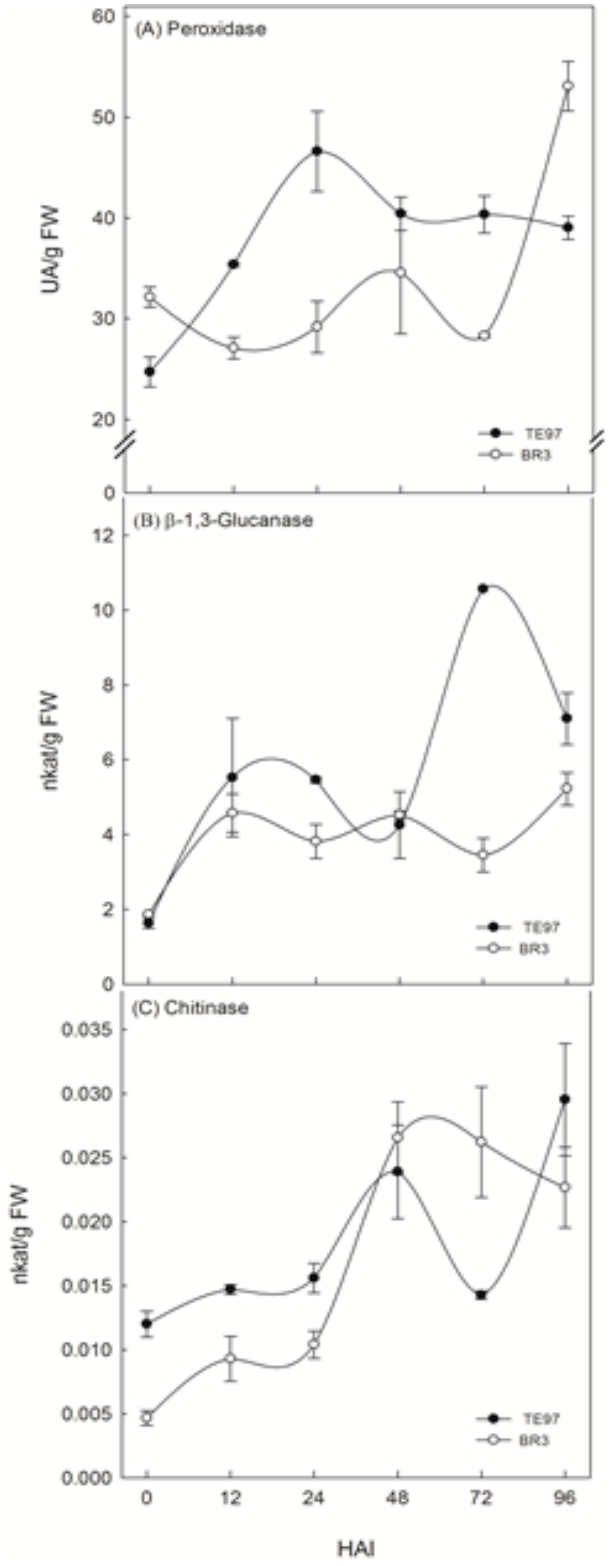

Figure 3: Kinetics of the PR-proteins POX (A), GLU (B), and CHI in the leaves of *C. gloeosporioides*-infected cowpea (*Vigna unguiculata*) genotypes TE97 (resistant, -●-) and BR3 (susceptible, -o-). Each data point represents the mean of three independent experiments ± standard error.

was observed from 0 (24.71 ± 1.48 UA g^{-1} FW) to 24 HAI (46.60 ± 3.98 UA g^{-1} FW) in the primary leaves of TE97 plants inoculated with *C. gloeosporioides*, whereas the BR3 plants exhibited decreased POX activity (Figure 3A). Moreover, at 12 and 24 HAI, the POX activity in TE97 was approximately 35.37 ± 0.19 and 46.60 ± 3.98, respectively, which was 30% and 60% higher than the corresponding values (27.09 ± 1.88 and 29.18 ± 2.58 AU g^{-1} FW, respectively) in the *C. gloeosporioides*-susceptible genotype BR3. In contrast, the BR3 genotype experienced a significant ($\rho \leq 0.05$) increase in POX activity at 96 HAI (53.08 ± 2.45 AU g^{-1} FW), which was nearly 1.7-fold higher compared with the basal (0 HAI) activity (32.14 ± 1.00 AU g^{-1} FW) and the POX activity observed in TE97 (39.30 ± 1.14 AU g^{-1} FW). A comparison of the POX activity in both inoculated genotypes showed that the activity in TE97 was more prominently induced than that in BR3 upon infection from 0 to 72 HAI.

The basal (0 HAI) GLU activities for the cowpea genotypes TE97 (1.63 ± 0.14 nkat/g FW) and BR3 (1.81 ± 0.09 nkat/g FW) were similar (Figure 3B). Upon infection with *C. gloeosporioides*, the activity increased in both genotypes between 0-24 HAI, with a significant difference in GLU activity between TE97 (5.46 ± 0.07 nkat/g FW) and BR3 (3.82 ± 0.46 nkat/g FW) at 24 HAI. However, the most noticeable effect on GLU activity occurred for TE97 at 72 (10.56 ± 0.00 nkat/g FW) and 96 HAI (7.10 ± 0.68 nkat/g FW), showing 3.1 and 1.4-fold higher increases compared with BR 3 (3.45 ± 0.45 nkat/g FW and 5.22 ± 0.44 nkat/g FW, respectively).

The chitinolytic activity of TE97 increased 2-fold from 0 HAI (0.012 ± 0.001 nkat/g FW) to 48 HAI (0.024 ± 0.004 nkat/g FW) and was consistently higher than that in BR3 at earlier stages (0-24 HAI) after inoculation with *C. gloeosporioides* (Figure 3C). Subsequently (48-72 HAI), the chitinolytic activity of BR3 was higher than that of TE97, and at 96 HAI there was no difference between the activities of the two inoculated genotypes. Overall, the CHI activity increased immediately after inoculation in TE97, whereas the induced response was delayed in BR3.

Discussion

Plants have developed a variety of complex mechanisms that involve the biosynthesis and accumulation of metabolites and novel and constitutive proteins that directly or indirectly function in the defense response to pathogens. In the present study, a significantly higher increase of SOD activity and a suppression of CAT activity were observed in TE97 than in the BR3 cowpea genotype within 24 h after inoculation (HAI) with *Colletotrichum gloeosporioides* (Figure 1A and 1B). Fang et al. [40] reported increased SOD activity from ~80% to ~200% of the control during the first 72 HAI in strawberry (*Fragaria ananassa*) leaves infected with *Colletotrichum fragariae*. However, those authors observed increased CAT activity after infection compared with the uninfected control at 24 HAI and according to the authors, this effect was not associated with the fungus infection [40]. Although a gradual increase in APX activity was observed in TE97 (Figure 1C), the total APX activity in BR3 was higher from 0 to 24 HAI compared with that in TE97. The increase in SOD activity, decrease in CAT activity and APX activity values below those of BR3 at 12 to 24 HAI are consistent with a greater accumulation of H_2O_2 within this period in the *C. gloeosporioides*-resistant cowpea genotype TE97 than in the susceptible BR3 genotype (Figure 1D), which also exhibits H_2O_2 accumulation, but to a lesser

extent. The results obtained from this study and a previous study [24] from our laboratory showed that the invasion of the cowpea primary leaves of BR3 and TE97 with *C. gloeosporioides* preferentially occurred in the leaf epidermal cells through penetration tubes that emerged from appressoria. Recently, it was shown that in susceptible and resistant coffee genotypes, hypocotyl penetration occurs directly in the epidermal cell cuticle through a thin infection hypha emerging from the melanized appressoria [15]. The primary leaves of TE97 showed enhanced penetration resistance to *C. gloeosporioides* associated with increased epidermal H_2O_2 accumulation beneath the appressoria and primary germ tubes, in addition to papillae formation [24]. The accumulation of H_2O_2 in infected cells and beneath appressoria was also observed in maize leaves during *Colletotricum graminicola* infection [41]. The interaction of cowpea x *C. gloeosporioides* studied in the present work and the macroscopic examination of the primary leaves of both cowpea genotypes revealed the presence of shrunken necrotic lesions, which are characteristic of anthracnose. In the genotype TE97, cell death was also observed, but only at a reduced percentage of the infection sites, indicating that cell death was restricted to the infection sites, as the small macroscopic lesions did not multiply in number neither increase in size during the 21-day experimental period, as previously observed [24]. In contrast, necrotic lesions increased in size and spread to the neighboring cells in the BR3 genotype, as observed in the *C. lagenarium* infection of susceptible melon leaves and other hosts infected with other *Colletrotrichum* species [42]. These results are also consistent with our previous observation that *C. gloeosporioides* establishes a compatible relationship and succeed in colonizing the BR3 cowpea genotype [24]. The restricted necrotic spots on the primary leaves of TE97 might be associated with HR in response to the accumulation of H_2O_2. As previously discussed, H_2O_2 accumulated biphasically with the time (0-12 and 48-72 HAI) in both cowpea genotypes, BR3 and TE97, after interaction with *C. gloeosporioides* (Figure 1D). H_2O_2 accumulation was confirmed through the microscopic examination of the DAB-treated primary leaves at 48-72 HAI around the papillae and subjacent to the appressoria of both genotypes at the infection sites (Figure 2). This observation suggests that the oxidative cross-linking of some constituents might strengthen the cell wall, forming a structural barrier against fungal ingress. Although papillae formation was observed, the pathogen infiltrated the host cells through penetration tubes formed in the appressoria, particularly in the BR3 genotype, for which higher numbers of appressoria with penetration tubes and necrotic lesions were observed during the experimental period (0 to 22 DAI) [24].

It has been suggested that H_2O_2 has direct antimicrobial effects and is involved in other defense responses, such as cell wall modification, lipid peroxidation, phytoalexin production, HR and defense-related gene activation, and serves as an intra- or intercellular signaling molecule in the activation of plant defense mechanisms against pathogen attack [1]. It was recently reported that treatment with bacterial and fungal elicitors (*F. oxysporum* cell wall elicitor, conserved epitopes of bacterial flagellin Flg22, elongation factor Elf26, and purified oligogalacturonides) induces apoplastic H_2O_2 production in *Arabidopsis thaliana* cell culture and that H_2O_2 generation is dependent on two cell wall peroxidases, PRX33 and PRX34 [43]. Vascular plants possess several genes encoding a number of POXs, and there might be some distinct physiological functions for each class in protecting the cell membrane against oxidative damage, which occurs in plants under biotic and abiotic stresses [4]. For example, the treatment of the primary leaves of cowpea cv. Vita 3 with 10 mM salicylic acid increased the total activity of the anionic POX isoform but not the cationic isoform [44]. POX could use H_2O_2 as a substrate for the oxidative polymerization of hydroxycinnamyl alcohol monolignols in the phenylpropanoid pathway to yield lignin [45], which reinforces the plant cell wall as a mechanism of disease resistance [46]. Equally important, some POXs exert antifungal activity against a variety of fungal species, including *Trichosporium vesiculosum*, *Macrophomina phaseolina*, *Coprinus comatus*, *Mycosphaerella arachidicola*, *Fusarium oxysporum* and *Botrytis cinerea* [12,13].

In the present study, the infection of the cowpea genotypes with *C. gloesporioides* induced POX activity in both resistant (TE97) and susceptible (BR3) genotypes. However, POX induction was temporally distinct because the TE97 POX activity increased immediately after inoculation, whereas in BR3, the activity was clearly retarded (Figure 3A). The differentiated POX kinetics might provide a mechanism for the defense strategy of TE97 against *C. gloeosporioides*. In a study where *Gossypium barbadense* cv. 7124 (resistant) and *G. hirsutum* cv. YZ-1 (susceptible) were infected with the highly aggressive defoliating fungus *Verticillium dahliae* strain V991, the resistant plants accumulated a higher level of POX activity than the susceptible plants in both the roots and stems [46].

The interaction of *C. gloeosporioides* with the resistant (TE97) and susceptible (BR3) cowpea genotypes induced significantly ($\rho \leq 0.05$) higher β-1,3-glucanase (GLU) activity at 24, 72 and 96 HAI in the resistant than in the susceptible genotype (Figure 3A). A second GLU response in TE97 showed 175% higher activity compared with that in BR3. An increase in chitinase (CHI) activity occurred in both cowpea genotypes from 0 to 48 HAI compared with the basal values at 0 HAI (Figure 3C). However, from 0-24 HAI, the CHI activity in TE97 was consistently higher ($\rho \leq 0.05$) than that in BR3. Moreover, a second increased CHI response was observed only in the resistant genotype TE97. The participation of GLUs in the plant defense response against fungal attack is associated with the ability of these enzymes to partially degrade the cell wall of the pathogens. The PR-proteins, GLU and CHI, often act synergistically to degrade chitin and β-1,3-glucans, respectively, which are the primary constituents of the fungal cell wall [16]. The inoculation of common bean (*Phaseolus vulgaris*) hypocotyls with a non-pathogenic species of *Rhizoctonia* (BRN) not only induced resistance to the virulent *Rhizoctonia solani* and *Colletotrichum lindemuthianum* but systemically induced POX, GLU and CHI compared with diseased control plants [47]. Daugrois et al. [48] reported that GLU and CHI activities were induced earlier in the incompatible interaction of *P. vulgaris* (cultivar Processor PrR) and *C. lindemuthianum* than in the compatible relationship with the cultivar Processor PrS. In this case, GLU and CHI activities were increased at 24 and 72 HAI, respectively, in the cv. resistant, whereas symptoms appeared at 96 HAI in the cv. susceptible. *In vivo* antifungal activity was demonstrated, as the results of a leaf disk bioassay revealed that the over-expression of a transgenic cacao class I CHI gene in *Theobroma cacao* significantly inhibited the growth of *C. gloeosporioides* and the development of leaf necrosis compared with control leaves that were wound inoculated with 5,000 spores [49]. In the incompatible interaction of the common bean (*Phaseolus vulgaris*, genotype SEL 1308) with race 73 of *C. lindemuthianum*, an accumulation of GLU transcripts (at 24, 48, 72, 96 HAI) was observed in the leaves, epicotyls and hypocotyls [50]. Similarly, the significant induction of CHI and GLU activity in the roots, cotyledons,

hypocotyls, and leaves of cowpea (cv. EPACE 10) seedlings infected with *Fusarium oxysporum* f. sp. *cubenses* and *F. oxysporum* f. sp. *Phaseoli* was observed at 72 and 96 HAI compared with control plants [51]. In the Acacia species, protection of the extrafloral nectar, which is rich in primary metabolites, from infestation with various phytopathogens is conferred through GLU and CHI activity, which potentially represents the most important prerequisite in this defensive mechanism, together with other PR-proteins [52].

Conclusion

In summary, the data presented in this study suggest that *C. gloeosporioides* induced oxidative burst in both cowpea genotypes TE97 and BR3 characterized by H_2O_2 generation. However, the higher H_2O_2 accumulation in TE97 together with the enhanced POX activity involved in lignification, as observed in coffee resistance to *Colletotrichum kahawae* [15], provides reinforcement of the plant cell wall for the restriction of the fungus spreading to neighboring cells. In addition, the induced levels of the PR-proteins GLU and CHI of TE97, compared with those of BR3, highlight the crucial role of these enzymes against phytopathogen attacks and confirm their participation in the defense strategy of the resistant cowpea genotype TE97 against *C. gloeosporioides*.

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