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H₂O₂ plays an important role in the lifestyle of *Colletotrichum gloeosporioides* during interaction with cowpea [*Vigna unguiculata* (L.) Walp.]

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ABSTRACT

Plant-fungus interactions usually generate H₂O₂ in the infected plant tissue. H₂O₂ has a direct antimicrobial effect and is involved in the cross-linking of cell walls, signaling, induction of gene expression, hypersensitive cell death and induced systemic acquired resistance. This has raised the hypothesis that H₂O₂ manipulation by pharmacological compounds could alter the lifestyle of *Colletotrichum gloeosporioides* during interaction with the BR-3-Tracueteua cowpea genotype. The primary leaves of cowpea were excised, infiltrated with salicylic acid (SA), glucose oxidase + glucose (GO/G), catalase (CAT) or diphenyliodonium chloride (DPI), followed by spore inoculation on the adaxial leaf surface. SA or GO/G-treated plantlets showed increased H₂O₂ accumulation and lipid peroxidation. The fungus used a subcuticular, intramural necrotrophic strategy, and developed secondary hyphae associated with the quick spread and rapid killing of host cells. However, CAT or DPI-treated leaves exhibited decreased H₂O₂ concentration and lipid peroxidation and the fungus developed intracellular hemibiotrophic infection with vesicles, in addition to primary and secondary hyphal formation. These results suggest that H₂O₂ plays an important role in the cowpea (*C. gloeosporioides*) pathosystem given that it affected fungal lifestyle during interaction.

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Introduction

One of the earliest defense reactions activated in plant tissues in response to pathogen attack is oxidative burst (Marino *et al.* 2012), characterized by rapid and transient release of reactive

oxygen species (ROS), represented by singlet oxygen (¹O₂), superoxide ion (O₂⁻), hydroxyl radical (OH•), and hydrogen peroxide (H₂O₂) (Shetty *et al.* 2008; Wang *et al.* 2010). ROS are produced by plant cells via the enhanced enzymatic activity of cell-wall-bound peroxidases, apoplastic amine oxidases,

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plasma-membrane-bound NADPH oxidases, respiratory burst oxidase homologues (RBOHs), glycolate oxidase, oxalate oxidase, xanthine oxidase and pathways such as photosynthesis, photorespiration, and respiration (Heller & Tudzynski 2011; Marino et al. 2012; Baxter et al. 2014). Enhanced ROS produced during the oxidative burst can function as cellular signaling molecules and antimicrobials in plant defense responses, but in excess may be detrimental to plants since these derivatives of molecular oxygen can damage cell constituents such as lipids, proteins, and nucleic acids (Wrzaczek et al. 2013; Baxter et al. 2014). Therefore, in addition to antioxidant compounds, several enzymes work together to tightly regulate the plant antioxidant network in order to maintain the steady-state level in plant cells (Ahmad et al. 2008), such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) (Singh et al. 2010), among others. Furthermore, it is suggested that plant hormones such as salicylic acid, jasmonic acid, and abscisic acid influence induction of ROS and antioxidants (Barna et al. 2012). H_2O_2 is the most chemically stable ROS. It has a direct antimicrobial effect and is involved in the cross-linking of cell walls, signaling, induction of gene expression, hypersensitive cell death and induced systemic acquired resistance (Quan et al. 2008). A number of studies have attempted to elucidate the role of H_2O_2 in different pathosystems. It has been reported that biotrophic pathogens are inhibited by H_2O_2 accumulation, whereas necrotrophic pathogens are favored by H_2O_2 production or even stimulate its production (Horbach et al. 2011; Barna et al. 2012). However, the role of H_2O_2 in the infectious process of hemibiotrophic pathogens is still elusive.

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., an anamorph of *Glomerella cingulata* (Ston.) Sp. & Schr., causes anthracnose in a wide range of plants including cowpea [*Vigna unguiculata* (L.) Walp.], a nutritious grain legume widely cultivated in parts of Asia, Oceania, Africa, South America, and particularly northeastern Brazil (Barreto et al. 2007). This fungus employs different strategies to invade host tissue, from intracellular hemibiotrophy, associated with large intracellular primary hyphae, to subcuticular/intramural necrotrophy, associated with narrower secondary hyphae that ramify throughout the host tissue (Münch et al. 2008; Talhinhas et al. 2011).

To address the role of H_2O_2 in different hemibiotrophic pathosystems, extracellular pharmacological compounds, capable of altering the levels of plant H_2O_2 in various pathosystems, have been applied (Jun et al. 2006; Shetty et al. 2007; Jindřichová et al. 2011). In the present study, we manipulated H_2O_2 concentrations in cowpea leaves to determine the causal relationship of H_2O_2 in the interaction between the BR-3-Tracuetea cowpea cultivar (hereafter, BR-3) and *C. gloeosporioides*. It is assumed that this fungus has a variable lifestyle, combining periods of biotrophy and necrotrophy at different H_2O_2 concentrations.

Material and methods

Biological materials

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. [teleomorph: *Glomerella cingulata* (Stonem.) Spauld. & von Schrenk],

previously identified by Barreto et al. 2007, was isolated and cultured on potato dextrose agar (PDA, Difco, Detroit, MI) under continuous fluorescent light at 22 °C. 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 conidial suspension was adjusted in sterile water to a known concentration after Neubauer chamber cell counting under a microscope (Olympus System BX60), and used as inoculum.

Cowpea seeds [*Vigna unguiculata* (L.) Walp.], BR-3-Tracuetea genotype, were provided by EMBRAPA-Meio-Norte (Piauí, Brazil). The seeds were surface-disinfected with 0.05 % (v/v) sodium hypochlorite (active chloride) for 3 min and then rinsed three times with sterile distilled water. They were then imbibed for 20 min in sterile distilled water and sown onto three superimposed Germtest® paper sheets (28 cm wide × 38 cm long) previously autoclaved (120 °C, 1.5×10^5 Pascal, 20 min) and humidified under sterile conditions (2.5 mL sterile distilled water per 1 g of paper) (Maia et al. 2010). Seeds ($n = 12$) were placed 5 and 2 cm from the top of the sheet and the sides of the paper, which was then rolled to form a cylinder. It was then stored vertically inside a plastic bag to maintain adequate humidity in a growth chamber under controlled conditions ($110 \mu E m^{-2} s^{-1}$, 13 h photoperiod, 27 ± 2 °C and $70 \pm 10\%$ relative humidity) for 4 d. Four-day-old seedlings were transplanted to plastic pots containing a 2.5 L nutrient solution (Hoagland & Arnon 1950), and modified as described by Silveira et al. 2001. The plants were kept under chamber conditions for a further 8 d.

Infiltration of pharmacological compounds

To investigate the role of H_2O_2 during different phases of *Colletotrichum gloeosporioides*-cowpea interaction, primary leaves were excised 8 d after transplantation (12 d after planting) and received infiltration injections of pharmacological compounds covering foliar areas of 1–2 cm² using a disposable, plastic needleless syringe (Zhang et al. 2004). The injections were applied to two regions on each side of the abaxial leaf blade, separated by the main vein. To increase H_2O_2 levels, cowpea leaves were either infiltrated with salicylic acid (SA) at 1.0, 2.5 or 5.0 mM, diluted in sterile distilled water or with *Aspergillus niger* glucose oxidase at 50.0, 100.0 or 200.0 U mL⁻¹ plus 2.0 mM D-glucose (GO/G) in 50.0 mM sodium phosphate buffer, pH 6.5 (PB). To scavenge H_2O_2 , leaves were infiltrated with bovine liver catalase (CAT) at 500.0, 1000.0 or 2000.0 U mL⁻¹ in PB. In addition, to inhibit ROS production, including H_2O_2 , leaves were infiltrated with diphenyliodonium (DPI), a suicide substrate inhibitor of NAD(P)H oxidase that suppresses the plant's capacity to perform oxidative burst (Küpper et al. 2002), at 1.0, 2.5 or 5.0 μM in 0.05 % dimethylsulphoxide (DMSO). Fresh (untreated) and different leaves infiltrated with sterile distilled water, PB and DMSO served as controls for the respective treatments. Moreover, pressure was applied with a needleless syringe to assess the effect on leaf H_2O_2 (non-infiltrated leaves). In previous studies, other concentrations of PB (10.0 mM and 25.0 mM) and DMSO (0.010% and 0.025%) were tested to evaluate which concentration did not interfere with H_2O_2 levels.

All of the solutions were sterilized by filtration using a 0.22 μm Millipore membrane filter. Treated leaves were then transferred to Petri dishes containing filter paper imbibed with distilled water and incubated in a dark chamber (28 ± 2 °C and 90 ± 10 % relative humidity).

H_2O_2 quantification

H_2O_2 concentration was measured spectrometrically, as previously reported (Gay et al. 1999). Infiltrated leaves were collected 2, 12, and 24 h after treatment (HAT) and 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 $12000 \times g$ 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 $(\text{NH}_4)_2\text{SO}_4$ + 25 mM H_2SO_4 . Solution B consisted of 0.125 mM xylenol orange + 100 mM sorbitol. 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 of fresh leaf mass (nmol H_2O_2 g^{-1} FM).

Histochemical location of H_2O_2 using DAB

DAB (3'-3'-diaminobenzidine; Sigma) was used to visualize H_2O_2 accumulation in cowpea leaves infiltrated with the pharmacological compounds (Thordal-Christensen et al. 1997). In addition to the leaves treated with SA, GO/G, CAT, and DPI with its respective controls, the DAB reaction was also assessed on leaves subjected to needleless syringe pressure (non-infiltrated leaves). DAB was dissolved in Milli-Q grade water initially adjusted to pH 3.0 with 1.0 N HCl and heated for 1 h at 50 °C, followed by the addition of 1.0 N NaOH to adjust pH to 4.0. Infiltrated and control leaves were then collected at 2 and 12 HAT and infiltrated with DAB by applying positive pressure (Lohaus et al. 2001). To that end, 100 mL of DAB were added to a Kitassato flask coupled to a vacuum pump, and the leaves were gently submitted to positive pressure of about 20 kPa for 10 min at 25 °C. Next, the leaves were rinsed exhaustively with distilled water, and 8 h later decolorized by incubation in 1.5 g L^{-1} trichloroacetic acid in 3:1 (v/v) mixture of ethanol + chloroform (TCA:ETOH:CHL solution) for 48 h.

Lipid peroxidation assay

Membrane damage was estimated in triplicate through malondialdehyde (MDA) formation using the thiobarbituric

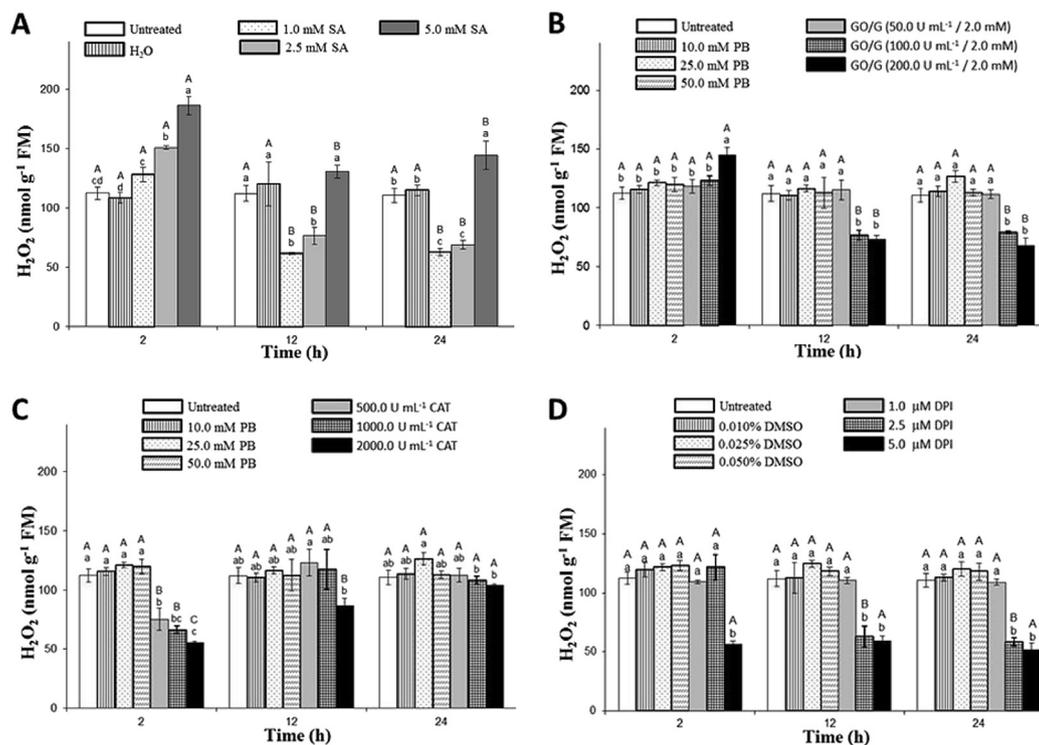


Fig 1 – Time course changing of H_2O_2 content in cowpea primary leaves infiltrated with SA (A), GO/G (B), CAT (C), DPI (D) and its respective controls. Basal H_2O_2 levels are those of untreated leaves. Each data point represents the mean of three independent experiments \pm standard error (bar). Different letters indicate significant differences ($p \leq 0.01$) in H_2O_2 contents between the treatments (Tukey's test). Small letters represent comparisons between the means of the different treatments on each specified time. Capital letters represent comparisons between the means of the same treatment during the experimental period.

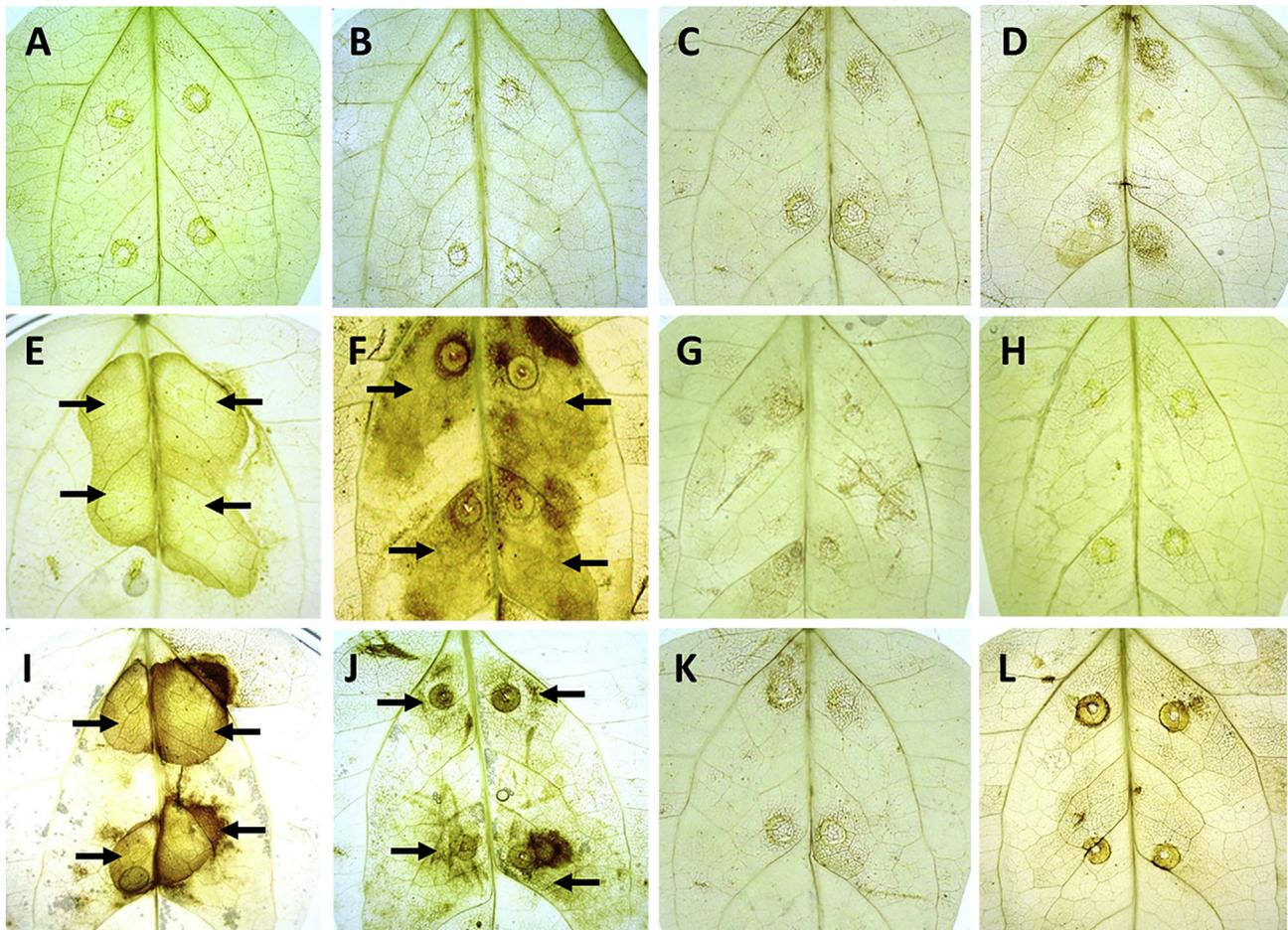


Fig 2 – Histochemical H_2O_2 detection (red-brown staining; arrows) after DAB staining in control cowpea primary leaves (non-infiltrated, H_2O , PB, and 0.05% DMSO infiltrated) and leaves infiltrated with 5 mM salicylic acid (SA); 2000.0 U mL^{-1} glucose oxidase plus 2.0 mM D -glucose (GO/G); 2000.0 U catalase (CAT); and 5.0 μM DPI. Non-infiltrated (A); infiltrated with H_2O (B); PB (C); and DMSO (D) leaves were examined at 2 HAT. SA (E, I); GO/G (F, J); CAT (G, K); DPI (H, L) infiltrated leaves were examined 2 and 24 HAT, respectively.

acid (TBA) method, as described by [Peever & Higgins 1989](#), with modifications. The infiltrated regions of the leaves were excised and collected at 2, 12, and 24 HAT with the pharmacological compounds and the tissues homogenized (1:3, m/v) in 1.0% TCA. The mixture was centrifuged at $12000 \times g$ for 15 min, at 4°C , and 1.0 mL of the supernatant was added to 3 mL of 0.5% (m/v) TBA containing 20% (m/v) TCA. The mixture was heated for 2 h at 95°C and cooled in an ice bath. It was then centrifuged at $9000 \times g$ for 10 min at 25°C and the supernatant absorbencies measured at 532 and 660 nm. After the non-specific absorbance (A_{660}) was subtracted, MDA concentration was estimated using its molar extinction coefficient ($155 \text{ M}^{-1} \text{ cm}^{-1}$) ([Heath & Packer 1968](#)), and expressed as nmol MDA per gram of fresh leaf mass ($\text{nmol MDA g}^{-1} \text{ FM}$).

Inoculation and light microscopy

Two hours after infiltration with the pharmacological compounds, cowpea primary leaves were inoculated with *Colletotrichum gloeosporioides* by dripping 50 μL of the spore suspension (4×10^5 cells mL^{-1} in sterile distilled water) onto

the infiltration region at the right of the main vein of the leaf on the adaxial side. Sterile Milli-Q grade water was dripped onto each infiltration region, but at the right of the main vein of the leaf, as control. The leaves were then transferred to Petri dishes and incubated, as previously described. The primary leaves were excised 12, 24, 48, and 72 h after inoculation (HAI) and used for analyses.

Light microscopy analyses were conducted after the infiltrated cowpea leaves were decolorized by incubation in a TCA:ETOH:CHL solution for 48 h with at least three different bleaching solutions. To visualize fungal structures, the depigmented leaf pieces were further stained with 0.5 g L^{-1} aniline blue in lactophenol for 2–3 min at 70°C ([Balows et al. 1991](#)). The fungal structures were strongly stained blue. To examine cellulose and chitin-bearing fluorescent structures, the decolorized tissues were first treated with 100 g L^{-1} KOH followed by 1.0 g L^{-1} (m/v) calcofluor white MR2 (Sigma). The leaf pieces were mounted on a microscope glass slide and covered with a glass coverslip onto which were added a few drops of glycerol ([Mlčková et al. 2004](#)). Light microscopic examinations were conducted with an Olympus System BX60 microscope

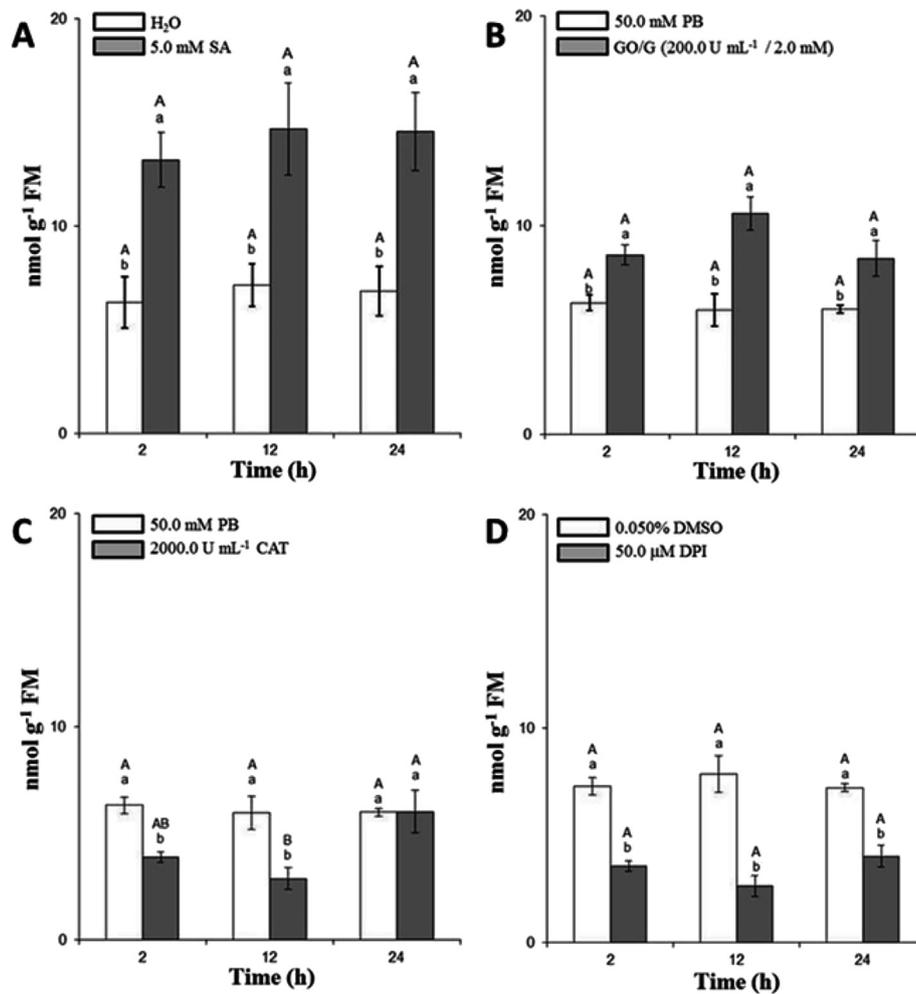


Fig 3 – Malondialdehyde (MDA) content in cowpea primary leaves infiltrated with SA (A), GO/G (B), CAT (C), DPI (D) and its respective controls. Each data point represents the mean of three independent experiments \pm standard error (bar). Different letters indicate significant differences ($p \leq 0.01$) in MDA content between treatments (Tukey's test). Small letters represent comparisons between the means of different treatments on each specified time. Capital letters represent comparisons between the means of the same treatment during the experimental period.

and fluorescence collected at 590 nm with the aid of a BX-FLA Olympus System Attachment. Images were acquired with a PM-20 Olympus photomicrograph system. The experiment was conducted in triplicate.

Statistical analysis

Three independent biological experiments were carried out in each test. Lipid peroxidation and quantitative production of H₂O₂ were subjected to analysis of variance followed by Tukey's test. Differences were considered significant at $p \leq 0.01$.

Results

H₂O₂ accumulation after pharmacological compound infiltration

The cowpea (BR-3 genotype) primary leaves were initially infiltrated with 1.0, 2.5, and 5.0 mM salicylic acid (SA), in order to

determine which concentration induces the highest accumulation of H₂O₂ without causing leaf damage. The H₂O₂ level at 2 HAT increased around 1.22 and 1.72-fold ($p \leq 0.01$) when the leaves were treated with SA at 2.5 mM and 5.0 mM, respectively, compared to untreated and H₂O treated controls (Fig 1A). At 12 and 24 HAT, although there was a significant decrease ($p \leq 0.01$) in H₂O₂ levels in the leaves treated with 5 mM SA, they were still significantly higher ($p \leq 0.01$) than those of control plants at 2 and 24 HAT. Treating cowpea leaves with 200.0 U mL⁻¹ GO + 2.0 mM G (GO/G) significantly ($p \leq 0.01$) increased the H₂O₂ level compared to controls at 2 HAT, but not at 12 and 24 HAT (Fig 1B), when the H₂O₂ levels were even lower in relation to those of untreated and PB controls. These results were in agreement with histochemical detection of H₂O₂ accumulation by DAB, indicated by the reddish-brown stain of the leaves, for SA-treated (Fig 2E and I) and GO/G-treated (Fig 2F and J) cowpea leaves when compared to controls (Fig 2A and B). Thus, the cowpea leaves were treated with 5.0 mM SA and 200.0 U mL⁻¹ GO + 2.0 M G for the ensuing experiments.

When the cowpea leaves were infiltrated with CAT as an H_2O_2 scavenger, H_2O_2 concentration ($p \leq 0.01$) decreased 2.15 and 1.35-fold in the CAT-treated leaves (2000.0 U mL^{-1}), at 2 and 12 HAT, respectively (Fig 1C). However, at 24 HAT, the H_2O_2 level increased to basal levels. For the DPI-treated ($5.0 \mu\text{M}$) leaves there was a 2.2-fold decrease ($p \leq 0.01$) in H_2O_2 level at 2 HAT, which persisted up to 24 HAT, when compared with DMSO controls (Fig 1D). Both removal and generation inhibition of H_2O_2 by CAT and DPI infiltration, respectively, were also evident by the lack of DAB staining (Fig 2G–H and K–L). The leaf infiltrations with water, PB and DMSO did not cause changes in H_2O_2 accumulation compared with the non-infiltrated leaves (Fig 1 and 2A–D).

Lipid peroxidation

The possible oxidative damage of the cell membrane, leading to lipid peroxidation, was studied by measuring the

malondialdehyde (MDA)-thiobarbituric acid (TBA) complex (MDA-TAB complex) concentration in infiltrated leaves collected at 2, 12, and 24 HAT. The MDA-TAB complex levels doubled in the leaves treated with 5.0 mM SA (Fig 3A) and increased, on average, 1.36-fold in the leaves treated with GO (200.0 U mL^{-1}) + G (2.0 M) compared to controls (Fig 3B). On the other hand, treating the cowpea leaves with CAT and DPI significantly ($p \leq 0.01$) decreased the MDA-TAB complex contents 2.15-fold, on average, when compared with control leaves (Fig 3C and D), except for CAT treatment at 24 HAT.

Effect of H_2O_2 on *Colletotrichum gloeosporioides* infection strategy

To analyze the effect of H_2O_2 on the infection process of *Colletotrichum gloeosporioides* in cowpea (BR-3 genotype), SA, GO/G, CAT, DPI and the controls, previously infiltrated leaves were inoculated 2 h later with fungal conidial suspensions and

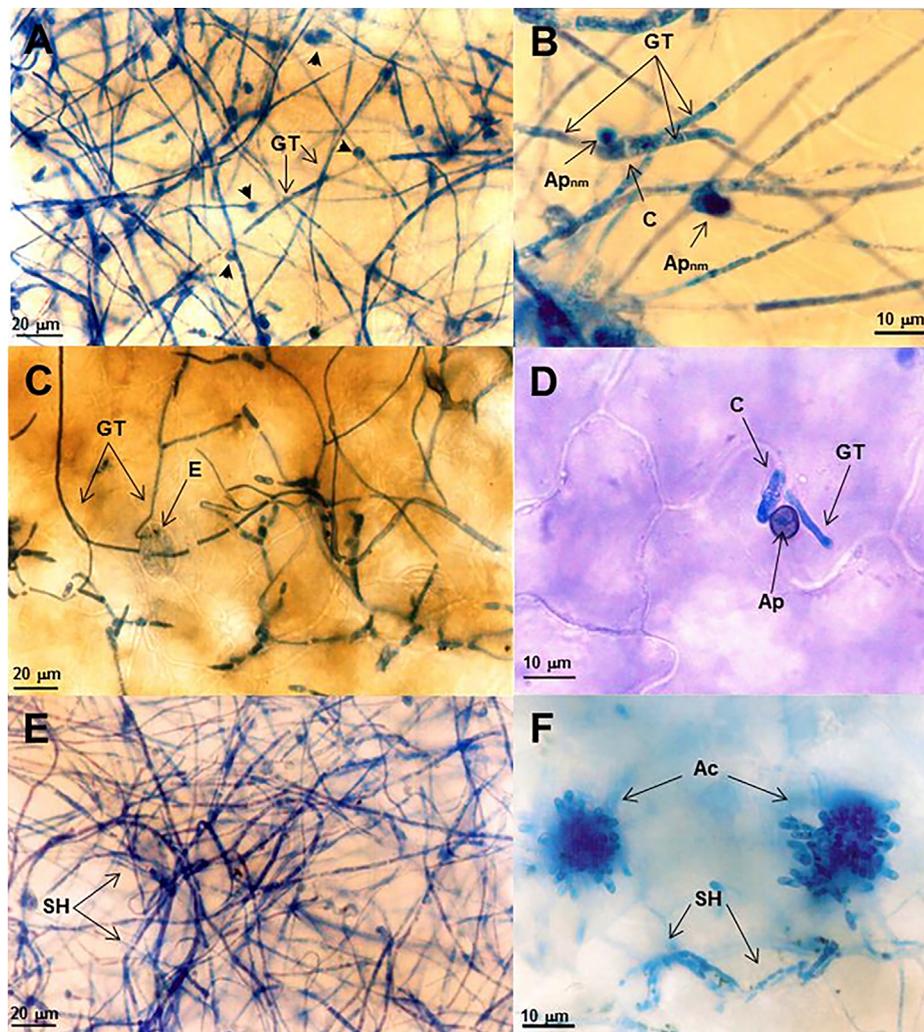


Fig 4 – Details of the infection process of *C. gloeosporioides* in cowpea primary leaves. (A): Germinating conidia (C), long germ-tube (GT) formation and nonmelanized appressoria (arrow heads) in SA infiltrated leaves within 12 HAI. (B): Detailed view of septate conidia (C), multiple long germ-tubes and nonmelanized appressorium (Ap_{nm}) formation in SA infiltrated leaves 24 HAI. (C): Detailed view of indirect penetration of the germ-tube through stomata in SA infiltrated leaves within 24 HAI. (D): Germinated conidia and appressorium (Ap) formation in GO/G infiltrated leaves at 12 HAI. (E): Secondary hyphae (SH) colonizing adjacent host cells in SA infiltrated leaves within 24 HAI. (F): Secondary hyphae (SH) anastomosis and acervuli (Ac) formation in SA infiltrated leaves within 48 HAI. Staining was done with aniline lactophenol blue.

collected 12, 24, 48, and 72 h after spore inoculation (HAI). None of these infiltrated compounds damaged the healthy cowpea leaves.

In the SA infiltrated leaves inoculated with *C. gloeosporioides* spores, the presence of germinated conidia was observed on the foliar cuticle, which became septate and formed long germ-tubes at 12 HAI followed by the formation of immature non melanized appressoria (Fig 4A, B). The fungus attempted to gain entry into the leaves through stomatal openings at 24 HAI (Fig 4C) and slowly colonized the mesophyll without initially producing visible symptoms. Long germ-tube formation was also observed on GO/G-treated leaves that formed melanized subglobose appressoria at 12 HAI (Fig 4D). However, although the presence of melanized appressoria and penetration pegs on GO/G-treated leaves was observed, the presence of biotrophic spherical vesicles was not detected. At 24 HAI, in both SA and GO/G-treated leaves, the fungus used a subcuticular, intramural necrotrophic strategy, forming secondary hyphae (5 μ m) associated with quick spreading and rapid killing of the host cells (Fig 4E). At 48 HAI, secondary hyphae invariably anastomosed with others, forming an inter connected network that developed asexual fruiting bodies (acervuli) surrounded by copious amounts of conidia (Fig 4F). Acervuli formation increased significantly within 72 HAI and was accompanied by intense conidial spreading. In SA and GO/G-treated leaves inoculated with *C. gloeosporioides*, fungal intracellular hemibiotrophic structures, such as infection

vesicles or primary hyphae, were not observed in the plant cells (Fig 4).

CAT and DPI infiltrations resulted in changes in the infection process of *C. gloeosporioides* from a necrotrophic to intracellular hemibiotrophic lifestyle. A similar strategy was also observed in all control treatments, including in H₂O-infiltrated leaves (Figs 5 and 6). After CAT and DPI treatments, which decrease leaf H₂O₂ concentration, conidia produced a septum across the middle of the spore and generated multiple short or long germ-tubes from which a number of appressoria were differentiated within 12 HAI (Fig 5A). By contrast, in control treatments only one melanized appressorium formation per short germ-tube was observed (Fig 5B). In CAT and DPI treatments an internal light spot was visible in melanized appressoria at 24 HAI, indicating that penetration pegs swelled to form a spherical infection vesicle (13–30 μ m) that developed bulbous lateral lobes, generating large multiseptate primary hyphae (5 μ m) (Fig 5C–D). These fungal structures grew intracellularly and characterized the biotrophic phase of infection. In control treatments, appressorium, and primary hyphae formation was observed within 48 HAI (Fig 6A–D). Secondary hyphae and acervuli were also produced within 48 HAI in CAT and DPI infiltrated leaves and at 72 HAI in H₂O, PB, and DMSO- treated leaves, characterizing the necrotrophic phase (Fig 6E–F). Cowpea (BR-3 genotype) leaf infiltration with H₂O, PB or DMSO followed by *C. gloeosporioides* inoculation caused no visible changes in fungal behavior

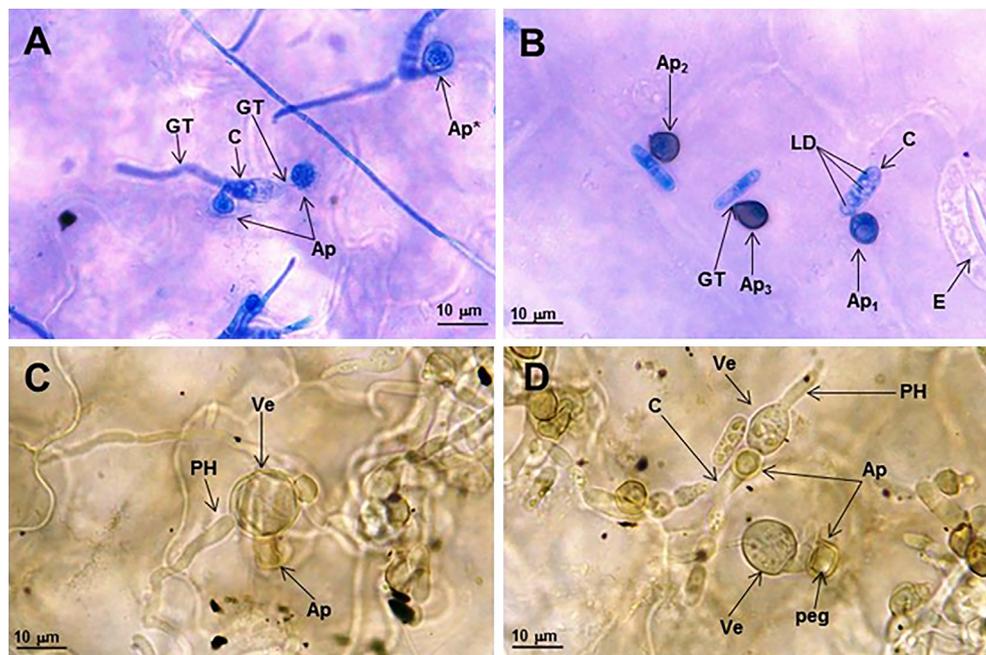


Fig 5 – Micrographs showing infection structures of *C. gloeosporioides* in cowpea infiltrated leaves. (A): Germinated conidium (C), germ-tubes (GT), and appressorium (Ap) formation in CAT treated leaves within 12 HAI. Ap* represents appressorium becoming melanized. (B): Germinated conidia and appressorium formation in leaves infiltrated with H₂O at 12 HAI. Ap₁, Ap₂ and Ap₃ represent the gradual process of appressorium melanization. Lipid droplets (LD) can be observed into conidia during germination and appressorium maturation (melanization). (C): Spherical infection vesicle (Ve) and primary septate hyphae (PH) formation in CAT treated leaves at 24 HAI. (D): Detailed view of penetration peg (peg), spherical infection vesicles (Ve), and primary septate hyphae (PH) in DPI treated leaves within 24 HAI. Staining was done with aniline lactophenol blue (A and B) and with calcofluor (C and D).

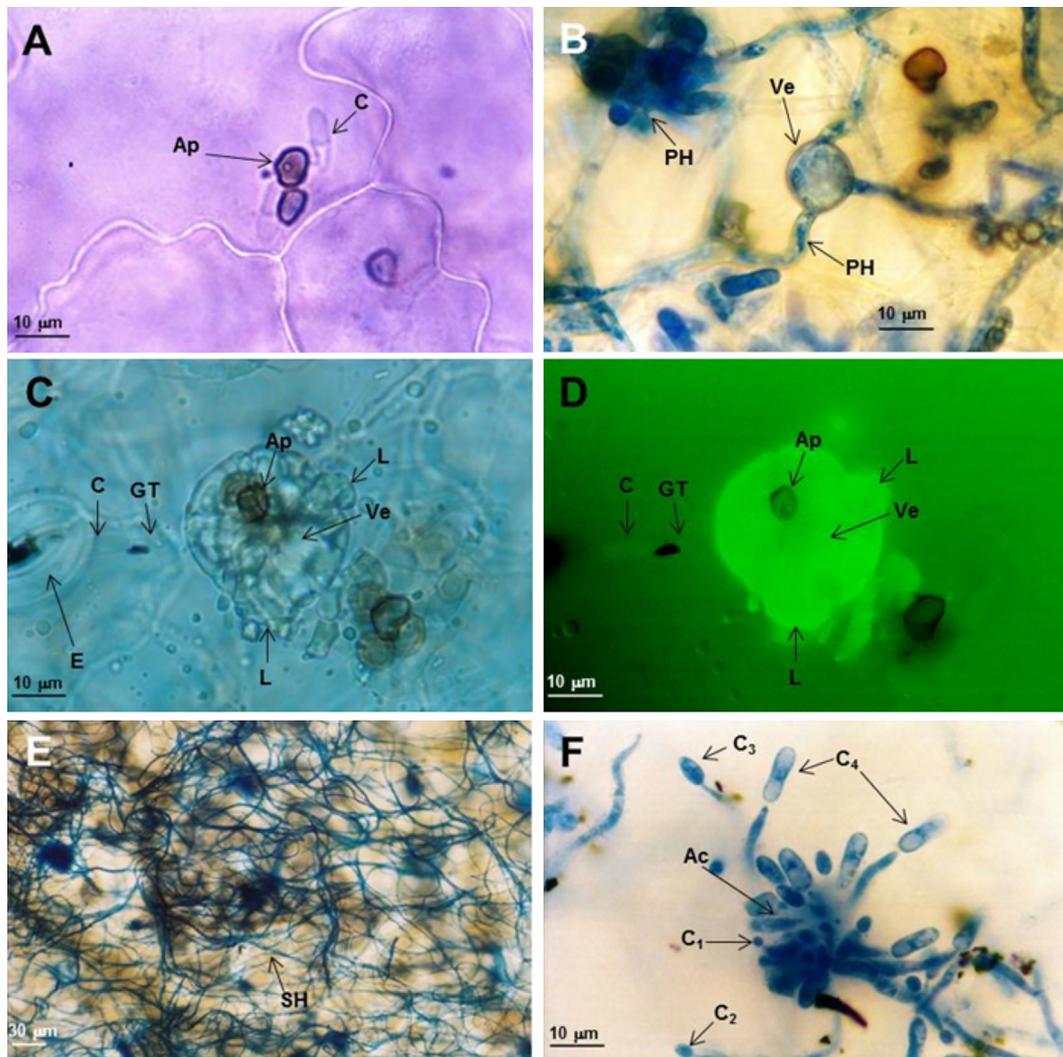


Fig 6 – Details of intracellular hemibiotrophic lifestyle of *C. gloeosporioides* in cowpea leaves infiltrated with H_2O (A–D) and CAT (E–F) within 48 HAI. (A): Germinated conidia (C) and appressorium (Ap) with penetration peg formation. (B): Details of spherical infection vesicle (Ve) and primary hyphae (PH). (C–D): Infection vesicle (Ve) with lateral lobes (L) and germ-tube (GT) inside the primary leaves. (E): Secondary hyphae (SH). (F): Acervuli (Ac). C₁, C₂, C₃, and C₄ represent the gradual development of conidia in acervuli conidiophores. Staining was done with aniline lactophenol blue (B, E, and F) and with calcofluor (A, C, and D).

compared with untreated leaves inoculated with the fungus (data not shown). Thus, at the much lower H_2O_2 concentration promoted by CAT and DPI treatments, in relation to physiological level, *C. gloeosporioides* infected the cowpea leaves employing an intracellular hemibiotrophic strategy with the formation of biotrophic vesicles in the primary leaves.

Discussion

Plants are continuously threatened by a variety of pathogens. To defend themselves against these attacks, they have evolved a number of constitutive and inducible mechanisms, one of which is the rapid and transient accumulation of H_2O_2 . Experimental evidence has proven that H_2O_2 plays an important role in plants under biotic stresses both as a signaling molecule that triggers a series of cellular responses and as

an antimicrobial agent (Quan et al. 2008; Shetty et al. 2008). In the present study, we investigated the role of H_2O_2 in the lifestyle of the hemibiotrophic pathogen *Colletotrichum gloeosporioides* in cowpea (BR-3 genotype) by altering leaf H_2O_2 concentration *in situ* after infiltration with salicylic acid (SA), glucose oxidase + glucose (GO/G), catalase (CAT) or diphenyliodonium chloride (DPI), followed by spore inoculation on the adaxial leaf surface.

SA infiltration of cowpea primary leaves increased both H_2O_2 concentration (Fig 1A; 2E and I) and lipid peroxidation (Fig 3A). Generally, H_2O_2 synthesis occurs at the same time as SA accumulation and both these molecules play a critical role in activating the hypersensitive reaction (HR) and systemic acquired resistance (SAR) (Hayat et al. 2010), which are key plant responses to pathogen attack. H_2O_2 and SA constitute a self-amplifying system where H_2O_2 induces SA accumulation and SA enhances H_2O_2 levels (Van Camp et al. 1998). In

addition, increased SA in plants causes inhibition of CAT and ascorbate peroxidase (APX), enzymes responsible for H₂O₂ degradation (Apel & Hirt 2004). Microscopic analysis of SA-treated leaves revealed the presence of immature nonmelanized appressoria (Fig 4A, B), since they did not have darkly melanized cell walls containing a basal penetration pore (Kleemann et al. 2008), as represented in Figs 5D and 6A. Moreover, the fungus infected the cells by penetrating through stomata, characterizing an indirect process (Fig 4C) that is not usually observed in the cowpea x *C. gloeosporioides* pathosystem (Barreto et al. 2007). Appressorial melanization is required for the infection of various fungal genera, including *Colletotrichum* (Deising et al. 2000; Münch et al. 2011). In the present study, appressorial melanization was observed in all treatments (Fig 5B) except in SA-treated plants, where increased H₂O₂ levels (Figs 1A, 2E and 2I) and lipid peroxidation (Fig 3A) were observed. It is suggested that the increase in H₂O₂ levels induced by SA infiltration might have caused intensive lipid peroxidation and damage to the fungal peroxisomes, interfering in the usual melanin synthesis of fungal appressoria (Fig 4B). Both germinating conidia and premature appressoria contain abundant lipid droplets that disappear during appressorial maturation, indicating lipolysis inside the conidial and appressorial cells (Asakura et al. 2012). Peroxisome integrity in *Colletotrichum lagenarium* is a prerequisite for fungal pathogenicity (Kimura et al. 2001). Deletion of genes (*pex6*, *pex13* or *mfe1*) related to peroxisomal metabolism, including β -oxidation, suppressed lipid breakdown and the melanization process in *Colletotrichum orbiculare* and *C. lagenarium* (Kimura et al. 2001; Asakura et al. 2012). Nevertheless, the fungus infected the leaf cells through stomata, forming secondary hyphae that characterize a subcuticular, intramural necrotrophic infection strategy followed by vigorous acervuli formation (Fig 4E–F). When the fungus employs this infection strategy, the penetration hyphae do not immediately enter the lumen of the cell; instead, they develop under the cuticle inside the periclinal and anticlinal epidermal cell walls (O'Connell et al. 2000). The secondary hyphae produced are specialized in toxin and hydrolytic enzyme secretion and their high area/volume ratio and thinner cell walls collaborate to maximize the nutritional process (Perfect et al. 2001; Münch et al. 2008). Thus, this lifestyle is marked by intra- and intercellular rapid hyphal ramification, causing cell death and dissolution of the cell walls during the infection process (O'Connell et al. 2000).

GO/G -infiltrated leaves also showed increased H₂O₂ levels, particularly at 2 HAT (Fig 1B; 2F and J), but only at the highest concentrations used, although it was significantly lower when compared with that of SA-infiltrated leaves at the same time. Enhanced lipid peroxidation from 2 to 24 HAT (Fig 3B) was also observed, but again much lower than that caused by SA. Transgenic plants of the genus *Arabidopsis* infiltrated with GO/G also showed increased H₂O₂ production both in the infiltrated region and those infected with *Pseudomonas syringae* pv. Tomato (Grant et al. 2000). Likewise, GO/G infiltration in *Arabidopsis* into small regions of leaves caused rapid H₂O₂ accumulation and triggered SAR in detached infiltrated leaves (Alvarez et al. 1998). In contrast to SA treatment, although there was long germ-tube formation in GO/G-treated leaves, appressorial formation was accompanied by melanization

(Fig 4D). As previously described (Perfect et al. 1999; Wharton & Diéguez-Uribeondo 2004; Rawlings et al. 2007) during this process, the conidium adheres to the cuticle and germinates, producing a germ-tube that differentiates into melanized appressoria. After melanization, a narrow penetration hypha emerges at the base of the appressorium and subsequently penetrates directly into the cuticle, forming biotrophic vesicles and primary hyphae (Münch et al. 2008). Nevertheless, in GO/G treatments, after direct penetration by melanized appressoria, the fungus developed a subcuticular, intramural necrotrophic lifestyle followed by secondary hyphae and vigorous acervuli formation, similarly to SA-treated leaves (Fig 4E–F). Thus, the increase in H₂O₂ in cowpea BR-3 genotype leaves induced by SA, and GO/G infiltration leads to the use of a necrotrophic infection strategy by *C. gloeosporioides*. Enhancement of H₂O₂ levels accompanied by increasing fungal virulence was also observed in other necrotrophic and hemibiotrophic pathosystems. The necrotrophic fungus *Botrytis cinerea* is virulent when there is strong oxidative burst (Schouten et al. 2002) and becomes avirulent in HR-deficient *Arabidopsis* mutants (Govrin & Levine 2000) and SOD-deficient *Phaseolus vulgaris* (Rolke et al. 2004). Likewise, in *Leptosphaeria maculosa*, a hemibiotrophic fungus with a short biotrophic phase, the infiltration of H₂O₂ resulted in a significant increase in *Brassica napus* lesions (Jindřichová et al. 2011). These data are consistent with those on interaction between tomato and *Colletotrichum coccodes*, which secretes ammonium ions to increase ROS content and thus improve virulence (Alkan et al. 2009).

On the other hand, CAT and DPI -infiltrated leaves showed decreased H₂O₂ levels (Fig 1C–D), which was corroborated by weak DAB staining of cowpea leaves (Fig 2G, H, K and L). A decrease in lipid peroxidation was also observed (Fig 3C–D). Both CAT and DPI treatments resulted in enhanced pathogen growth and did not interfere with the infection progress of *C. gloeosporioides*. Similar results were reported when CAT and DPI induced a decrease in DAB staining resulting from H₂O₂ depletion in *Arabidopsis* (Grant et al. 2000), *Lycopersicon esculentum* (Borden & Higgins 2002), *Phaseolus aureus*, and *Vicia sativa* (Zhang et al. 2009).

In CAT and DPI treatments, the fungus showed an intracellular hemibiotrophic lifestyle, since it developed a penetration peg, spherical vesicles, and primary hyphae (Fig 5C and D), characterizing the biotrophic phase, also observed in all control treatments (Fig 6A–D). These structures can vary greatly in morphology and the complete cycle varies in duration from less than 24 h to over 3 d (Perfect et al. 2001). The spherical vesicles shown in this study are also found in *P. vulgaris* against *Colletotrichum lindemuthianum* (Rawlings et al. 2007) and in cucumber against *C. orbiculare* (Asakura et al. 2012). However, this structure differs morphologically from that of *Colletotrichum destructivum*, which does not develop spherically, but rather as swollen multilobed vesicles during infection (Shen et al. 2001). This biotrophic phase is asymptomatic and marked by invagination of the host cell plasma membrane around the infection vesicle and primary hyphae (Mendgen & Hahn 2002). After penetration, the membrane loses its functional integrity and the host cells degenerate and die, characterizing the beginning of the necrotrophic phase with rapid cell degradation (Perfect et al. 2001). In the present study, the necrotrophic phase was observed within

48 HAI, with secondary hyphae production (Fig 6E). A similar phenomenon occurred in the control treatments at 72 HAI. In the course of the intracellular hemibiotrophic infection process of cowpea leaves treated with CAT and DPI, mycelium dimorphism was observed between the primary (Fig 5C–D) and secondary hyphae (Fig 6E). As previously described (Heath & Skalamera 1997), the production of mycelium dimorphism is a typical feature of many hemibiotrophic fungi, including *C. destructivum*, *Colletotrichum truncatum*, *Colletotrichum sublineolum*, and *Magnaporthe grisea*. The secondary hyphae produced in the necrotrophic phase contain a high area/volume ratio and thinner cell walls when compared to primary hyphae (Perfect et al. 2001). In all treatments, the success of the colonization process was evidenced by the formation of various non-septate acervuli (Figs 4 and 6). By contrast, one or many septate melanized setae were observed in *C. lindemuthianum*, *C. destructivum*, *Colletotrichum linicola*, and *C. truncatum* acervuli (Latunde-Dada & Lucas 2007), differing from the fungus studied here. However, melanized septate acervuli were observed in compatible interaction in planta between *C. gloeosporioides* and the cowpea BR-3 genotype 16 d after inoculation (Barreto et al. 2007).

Conclusion

In summary, it was clearly shown that neither the increase nor decrease of H₂O₂ accumulation in the primary leaves of cowpea (genotype BR-3-Tracueteua), resulting from leaves infiltrated with salicylic acid (SA) or glucose oxidase + glucose (GO/G), and catalase (CAT) or diphenyliodonium chloride (DPI), respectively, followed by inoculation of *Colletotrichum gloeosporioides* spores on the adaxial leaf surface, prevented *C. gloeosporioides* entry into cowpea host cells. However, at higher H₂O₂ concentrations, the fungus adopts a subcuticular, intramural necrotrophic strategy, whereas at lower H₂O₂ concentrations it adopts an intracellular hemibiotrophic lifestyle to infect cowpea. These findings suggest that leaf hydrogen peroxide balance plays an important role in the cowpea-*C. gloeosporioides* pathosystem, given that it affected fungal infection strategy.

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