Infection Process and Host Defense Responses in Compatible and Incompatible Interactions between Cowpea (*Vigna unguiculata*) and *Colletotrichum gloeosporioides*

Author(s): A. L. H. Barreto, I. M. Vasconcelos, T. B. Grangeiro, V. M. M. Melo, T. E. Matos, Y. R. G. Eloy, C. F. Fernandes, D. C. Torres, F. R. Freire-Filho, F. C. O. Freire, and J. T. A. Oliveira

Source: *International Journal of Plant Sciences,* Vol. 168, No. 2 (February 2007), pp. 193-203
Published by: The University of Chicago Press
Stable URL: http://www.jstor.org/stable/10.1086/509608
Accessed: 29/05/2013 14:55

Int. J. Plant Sci. 168(2):193–203. 2007.

1058-5893/2007/16802-0006$15.00

INFECTION PROCESS AND HOST DEFENSE RESPONSES IN COMPATIBLE AND INCOMPATIBLE INTERACTIONS BETWEEN COWPEA (*VIGNA UNGUICULATA*) AND *COLLETOTRICHUM GLOEOSPORIOIDES*

A. L. H. Barreto,* I. M. Vasconcelos,† T. B. Grangeiro,‡ V. M. M. Melo,‡ T. E. Matos,† Y. R. G. Eloy,† C. F. Fernandes,§ D. C. Torres,‡ F. R. Freire-Filho,* F. C. O. Freire,‖ and J. T. A. Oliveira[1,]†

*Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)–Meio-Norte, Piauí, CEP 64006-220, Brazil; †Departamento de Bioquímica e Biologia Molecular, Laboratório de Proteínas Vegetais de Defesa, Universidade Federal do Ceará, Caixa Postal 6033, CEP 60450-970, Brazil; ‡Departamento de Biologia, Universidade Federal do Ceará, CEP 60451-970, Brazil; §EMBRAPA-Rondônia, CEP 78900-970, Brazil; and ‖EMBRAPA–Agroindústria Tropical, Ceará, CEP 60511-110, Brazil

The infection process of *Colletotrichum gloeosporioides* was examined by light microscopy on two cowpea genotypes, BR 3 Tracuateua and TE 97-411. In the susceptible genotype BR 3 Tracuateua, the pathogen acted as an intracellular hemibiotroph. Invasion of cowpea primary leaves occurred preferentially through the leaf epidermal cells by penetration tubes that emerged from appressoria. The penetration tube swelled within 48 h to form a spherical infection vesicle inside the epidermal cell in a biotrophic interaction. Subsequently, it became multilobed and multiseptated. By 4 d after inoculation, one or more large primary hyphae emerged from the lateral lobes and grew intracellularly and further colonized several adjacent epidermal host cells, characterizing the necrotrophic phase of infection. Primary leaves of the resistant cowpea genotype, TE 97-411, showed enhanced penetration resistance to *C. gloeosporioides* associated with higher epidermal H_2O_2 accumulation beneath appressoria and primary germ tubes, papilla formation, and increased phenylalanine ammonia-lyase activity, possibly related to accumulation of phenolic compounds and host cell wall lignification. Macroscopic examination of the primary leaves revealed the presence of shrunken necrotic lesions characteristic of anthracnose in infected BR 3 Tracuateua, whereas in the genotype TE 97-411, cell death was also observed but only in a reduced percentage of the infection sites. In summary, the results obtained in this study suggested that TE 97-411 is the resistant genotype to *C. gloeosporioides* compared with BR 3 Tracuateua because it developed more effective defense responses against the establishment of the pathogen.

Keywords: Colletotrichum gloeosporioides, cowpea, defense responses, infection process.

Introduction

Cowpea is an important food legume that provides calories, proteins, minerals, and vitamins (Maia et al. 2000) to a large number of people living in the drier regions of tropical and subtropical countries, particularly in parts of Asia, Oceania, the Middle East, Africa, and Central and South America (Singh et al. 2002). Unfortunately, cowpea is susceptible to a wide range of pests and pathogens that attack all plant parts during its life cycle. These include viruses, bacteria, fungi, nematodes, and insects that restrain production, particularly grain yield.

Fungi are among the most serious pathogens that infect cowpea. In the genus *Colletotrichum*, various species, including *C. dematium*, *C. destructivum*, and *C. gloeosporioides*, are considered major cowpea pathogens worldwide and cause important economic losses. Apart from the economic impact, *Colletotrichum* is used as a model system for studying infection processes. To infect their hosts, *Colletotrichum* species use different strategies, depending on the fungus species and the plant attacked. They can invade the host cells to establish intracellular hemibiotrophic infections or can employ a subcuticular, intramural strategy or, alternatively, a combination of both (Bailey et al. 1992). For example, *C. destructivum* isolates that attack cowpea and alfalfa, and *C. gloeosporioides*, which infects round-leaved mallow (*Malva pusilla*) leaves (Wei et al. 1997), were shown to employ an intracellular hemibiotrophic strategy (Latunde-Dada et al. 1996, 1997). In this mode of nutrition, the fungus has an initial biotrophic phase in which it feeds on living host cells, and the host is symptomless. This is followed by a destructive necrotrophic phase in which the fungus causes extensive degradation of host cells, and symptoms become visible. However, *C. capsici*, which causes brown blotch in cowpea (Emechebe 1981), invades cowpea tissues through an initial subcuticular, intramural, necrotrophic mode of infection. Accordingly, following penetration of the cuticle, this fungus does not immediately enter the cell lumen but develops beneath the cuticle within the periclinal and anticlinal walls of epidermal cells (Pring et al. 1995; O'Connell et al. 2000).

[1] Author for correspondence; e-mail jtaolive@ufc.br.

Manuscript received December 2005; revised manuscript received August 2006.

Isolates of *C. gloeosporioides* (Penz.) Penz and Sacc. cause anthracnose on a wide range of hosts, particularly in the tropics, where they affect fruits and legumes (Manners et al. 2000). It has been suggested that the cowpea anthracnose pathogen should be regarded as a form of *C. gloeosporioides* and not as the pathogen *C. lindemuthianum* associated to the anthracnose of *Phaseolus* beans (Emechebe and Florini 1997). This study investigated the infection process of one *C. gloeosporioides* isolate on cowpea leaves in compatible and incompatible interactions and, in addition, assessed immediate defense responses performed by the host.

Material and Methods

Biological Materials and Inoculation

The isolate of the fungus *Colletotrichum gloeosporioides* was cultured on potato dextrose agar (PDA, Difco, Detroit, MI) under continuous fluorescent lighting at 25°C. For molecular identification of the fungus isolate, its genomic DNA was purified from mycelium using a CTAB-based protocol (Warner 1996). The internal transcribed spacers (ITS1 and ITS2) and the 5.8S coding region of the nuclear ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR) using the primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GCAAGTAAAAGTCGTAACAAGA). Amplification reactions were performed in a final volume of 25 μL containing 300 ng of genomic DNA (template); 20 mM Tris–HCl; pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 100 μM of each dATP, dCTP, dGTP, and dTTP (Amersham Biosciences, São Paulo); 12.5 pmol of each primer; and 0.5 units of Taq DNA polymerase (Amersham Biosciences). PCR reactions were carried out in an MJ-Research (Watertown, MD) PTC-200 thermocycler programmed for an initial denaturation step (4 min at 94°C) followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The last cycle was followed by a final incubation of 10 min at 72°C. Control samples containing all reaction components except DNA were always used to test that no self-amplification or DNA contamination occurred. Amplification of the DNA band with the expected size was checked by analyzing a 2-μL aliquot of PCR reactions by 1.0% agarose gel electrophoresis (Sambrook et al. 1989). Once the specificity of the amplifications was confirmed, PCR products were purified from the remaining reactions using GFX PCR DNA and a Gel Band Purification kit (Amersham Biosciences). The concentration of the purified PCR products was determined by measuring the absorbance at 260 nm (A_{260}) of a 10-fold dilution. DNA sequencing was performed with the DYEnamic ET terminators cycle sequencing kit (Amersham Biosciences), following the protocol supplied by the manufacturer, and both strands were sequenced using the primers ITS4 and ITS5. Sequencing reactions were then analyzed in a MegaBACE 1000 automatic sequencer (Amersham Biosciences). The determined sequence was then compared to those already deposited in the GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990).

Spore suspensions of the fungi 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 and used as inoculum.

Seeds of cowpea genotypes resistant (TE 97-411 [hereafter, TE97]) and susceptible (BR 3 Tracuateua [hereafter, BR3]) to *C. gloeosporioides* were obtained from EMBRAPA–Meio-Norte (Piauí, Brazil). 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. They were kept at 27°–35°C in a greenhouse exposed to natural light and daily irrigated with autoclaved (120°C, 1.5 KGF, 20 min) water for up to 4 d after sowing. Subsequently, they were irrigated with five times diluted Hoagland and Arnon (1950) nutritive solution modified as described by Silveira et al. (2001). Ten days after they were sowed, healthy plantlets were selected and transferred to a growth chamber kept at 25°–30°C, 85%± 5% relative humidity with a 12-h photoperiod at an intensity of ca. 200 μmol s^{-1} m^{-2} photosynthetically active radiation. Two days later, the primary leaves were inoculated by applying two 25-μL-equidistant droplets of spore suspension (4×10^5 mL^{-1} in sterile distilled water) to each side of the adaxial leaf blade separated by the main vein. Control plants were inoculated with sterile water. Leaves were collected at 0, 12, 24, 48, 72, and 96 h after inoculation (HAI) to analyze phenylalanine ammonia-lyase activity and at 1, 2, 3, 4, 5, 6, 8, 11, 16, and 22 d after infection to carry out the microscopic analyses.

Light Microscopy

All light microscopy analyses were conducted after the cowpea leaves were decolorized by incubation in 1.5 g L^{-1} trichloroacetic acid (TCA) in a 3 : 1 (v/v) mixture of ethanol + chloroform for 48 h with at least three changes of the bleaching solution. To detect autofluorescence as an indicator of phenolic compound accumulation (Borden and Higgins 2002), the leaves were soaked in 0.1 M KH_2PO_4/K_2HPO_4 buffer at pH 9.0 for 24 h, and the leaf pieces with infection sites were placed on glass slides in a few drops of the phosphate buffer. Alternatively, phenolics were visualized by soaking the leaf pieces in 50-mM citrate buffer at pH 3.5 for 1 h, followed by staining with 0.5 g L^{-1} toluidine blue for 10 min. Phenolic compounds were visualized as violet blue structures. To detect callose (papillae) formation, after the leaf pieces were treated with phosphate buffer, as previously described, they were stained with a 0.1 g L^{-1} buffered solution of aniline blue for 2 h (Borden and Higgins 2002). 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} (w/v) calcofluor white MR2 (Sigma, São Paulo), whereas nonfluorescent structures were visualized by treatment of the same leaf tissues with 0.4 g L^{-1} Evans blue. Lignification was visualized after the decolorized leaf pieces were immersed in 70% (v/v) ethanol containing 10 g L^{-1} phloroglucinol for 16 h, which stains lignified cell walls red orange. Excess stain was washed out with Milli-Q grade water. The leaf piece was mounted on a microscope glass slide, cleared by drop-washing with concentrated HCl (ca. 2 min),

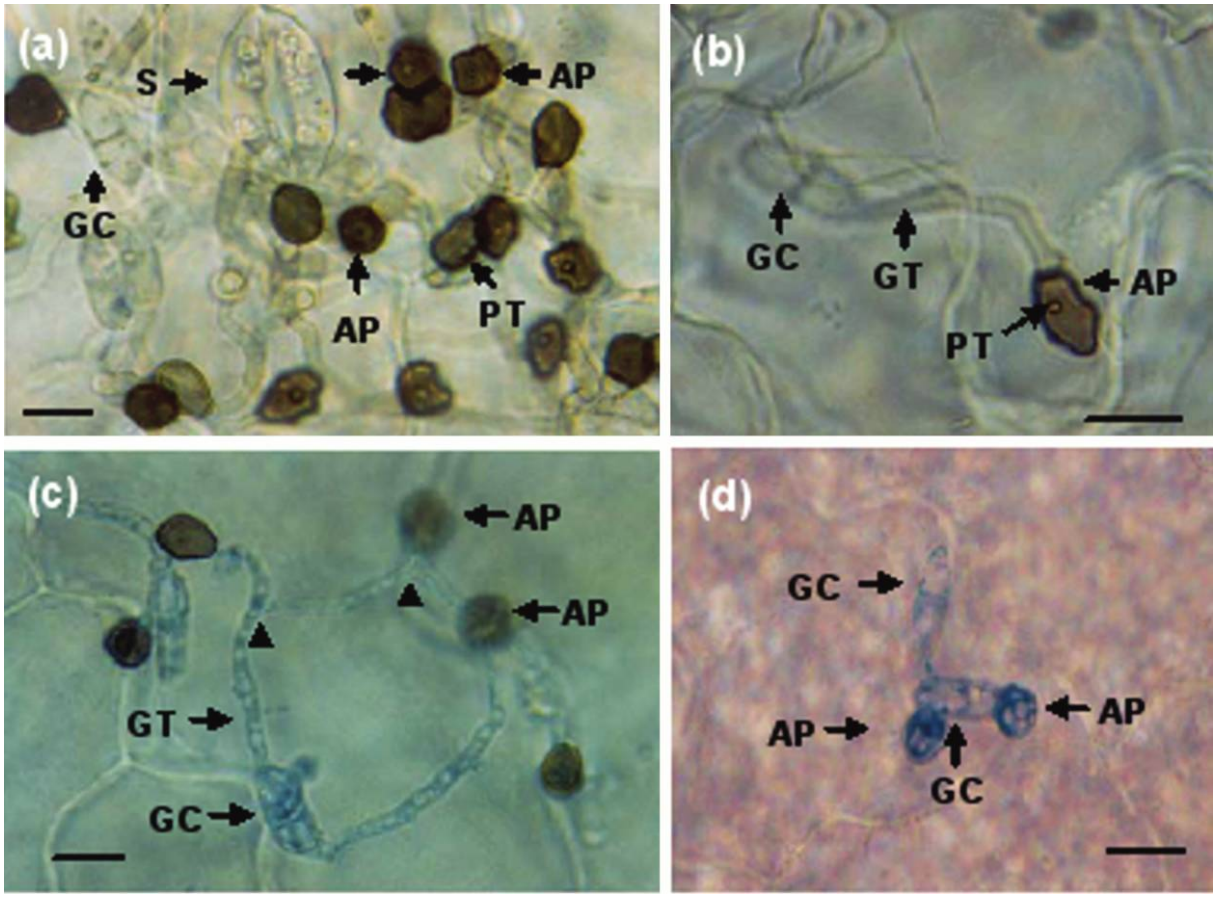

Fig. 1 Micrographs. *a*, Infection structures of *Colletotrichum gloeosporioides* in the primary leaves of BR3 cowpea genotype (compatible interaction) during the biotrophic phase 48 h after inoculation (HAI). *b*, A detailed view of an infection structure at 72 HAI. *c*, Germ tubes that ramified and produced multiple melanized appressoria or anastomosed (arrowheads) at 8 d after infection. *d*, Anastomosis of two conidia, which give rise to nonmelanized appressoria at 24 HAI. Appressorium (*AP*), germinating conidium (*GC*), germ tube (*GT*), penetration tube (*PT*), stoma (*S*). Staining was done with calcofluor and Evans blue (blue staining). Bars = 10 μm.

and covered with a glass coverslip to which a few drops of glycerol were added (Mlícková et al. 2004).

To detect H_2O_2 accumulation in the primary leaves, DAB (3′-3′-diaminobenzidine; Sigma) was infiltrated according to Thordal-Christensen et al. (1997). Stems of cowpea plantlets harvested at different periods after inoculation were cut 2 cm above the cotyledon insertion region, and the cut end of the upper plantlet part 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 at 50°C, followed by an addition of 1.0 N NaOH adjusted to pH 4.0. After 8 h treatment, leaves were excised and decolorized

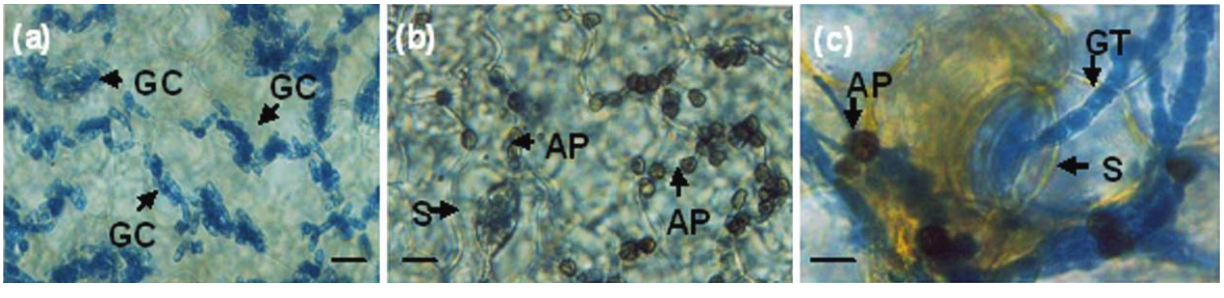

Fig. 2 Micrographs showing infection structures of *Colletotrichum gloeosporioides* in the primary leaves of TE97 cowpea genotype (incompatible interaction). *a*, Germinating conidia (*GC*) adhered preferentially above the spaces between epidermal cells and stoma (*S*) within 48 h after inoculation. *b*, Germinating conidia and appressoria (*AP*) within 6 d after infection. *c*, Detailed view of indirect penetration by the germ tube (*GT*) of *C. gloeosporioides* through stoma. Staining was done with calcofluor and Evans blue (blue staining) for *a* and *b* and aniline lactophenol blue for *c*. Scale bars = 20 μm for *a* and *b* and 10 μm for *c*.

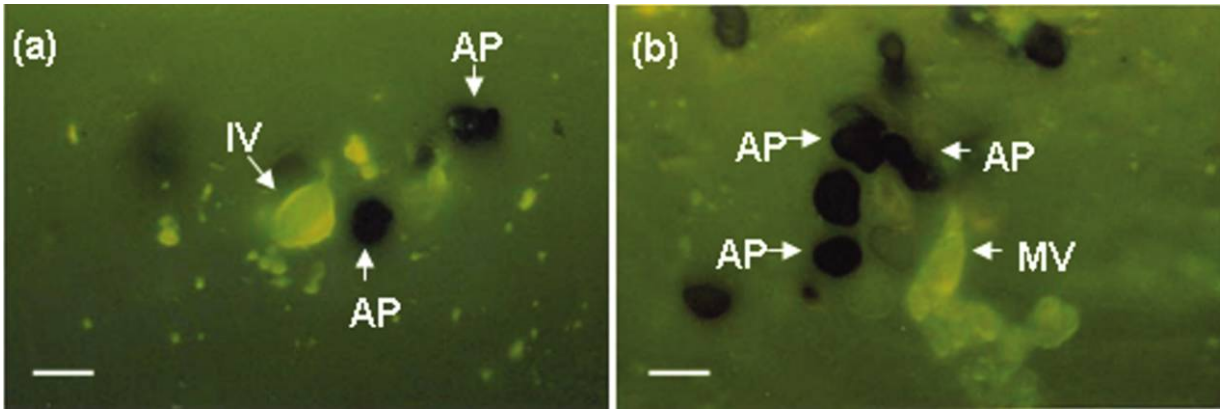

Fig. 3 Micrographs. *a*, Spherical infection vesicle (*IV*) with a short neck region (arrow) emerged from an appressorium of *Colletotrichum gloeosporioides*. *b*, Multilobed vesicle (*MV*) with lateral lobes inside the primary leaves of BR3 cowpea genotype within 48 h after inoculation. Staining was done with calcofluor and Evans blue. Scale bars = 10 μm for *a* and *b*.

with TCA/ethanol/chloroform as previously described. To visualize concomitantly the fungal structures, the DAB-treated leaf pieces were further stained in 0.5 g L^{-1} aniline blue in lactophenol for 2–3 min at 70°C (Balows et al. 1991). Fungal structures were strongly stained blue.

Light microscopic examinations were made with an Olympus System BX60 microscope and fluorescence collected at 590 nm with the aid of an Olympus System Attachment BX-FLA device. Images were acquired with Olympus photomicrography system PM-20. The experiments were repeated three times with three replications.

Phenylalanine Ammonia-Lyase Assay

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity was measured combining the methods previously described by Mori et al. (2001) and El-Shora (2002) with some modifications. Briefly, cowpea primary leaves were homogenized with a mortar and pestle in an extraction buffer (0.5 M Na-acetate buffer, pH 5.2, containing 0.5 M NaCl) in an ice bath. The homogenate was filtered through one layer of cheesecloth and centrifuged at 10,000 *g* for 20 min at 4°C. The supernatant was dialyzed exhaustively against the extraction buffer and used as a source of crude enzyme for assaying PAL activity. Protein was estimated by the method of Bradford (1976), using BSA as a standard. The reaction mixture (0.9 mL) contained 0.48 mL 0.1 M Tris–HCl buffer (pH 8.8), 0.02 mL 0.05 M β-mercaptoethanol, 0.2 mL 0.04 M L-phenylalanine, and 0.2 mL cowpea leaf extract. Incubation was performed at 30°C for 1 h, and the reaction stopped with the addition of 0.1 mL 6 N HCl. In the blank tube, L-phenylalanine was added after incubation and the addition of HCl. The reaction mixture was then centrifuged at 10,000 *g* for 10 min at 25°C, and the absorbance of the supernatant measured at 290 nm. One unit of enzyme activity equals the amount (pmol) of trans-cinnamic acid produced in 1 s and expressed per gram fresh leaf mass (pmol g^{-1} FM s^{-1}).

Results

To confirm the identity of the fungus species used in this work, the complete sequence of the ITS region of rDNA

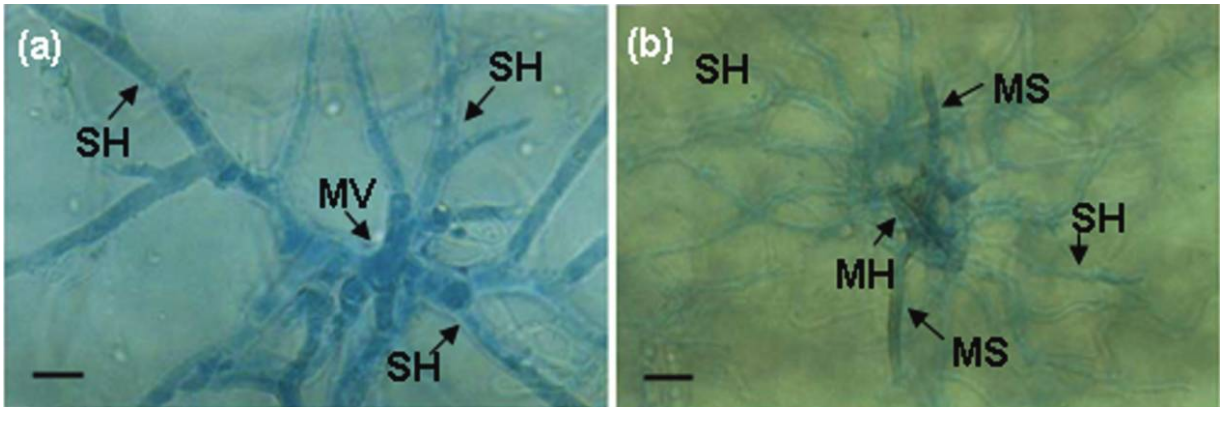

Fig. 4 Micrographs. *a*, Irradiation of secondary hyphae (*SH*) from a multilobed vesicle (*MV*) colonizing adjacent host cells in the primary leaf of TE97 genotype at 96 h after inoculation. *b*, Radiation of melanized secondary hyphae (*MH*) of *Colletotrichum gloeosporioides* emerging from the primary leaf cuticle of BR3 genotype and initial formation of acervulus, resulting from aggregation of secondary hypha (*SH*), showing two visible melanized setae (*MS*) at 16 d after infection. Staining was done with aniline lactophenol blue. Scale bars = 10 μm for *a* and 20 μm for *b*.

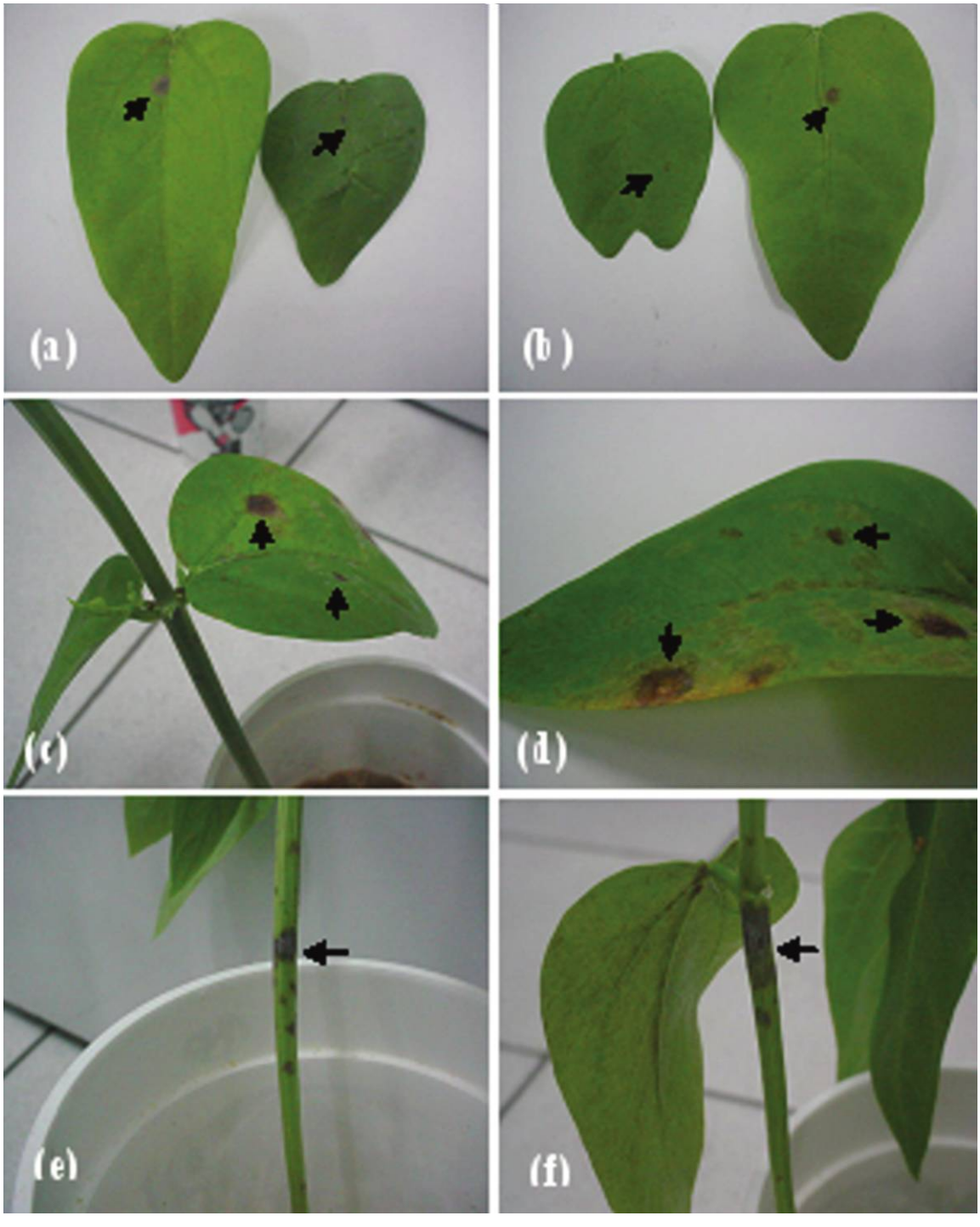

Fig. 5 Comparison (*a*, *b*) of the necrotic lesions (arrows) formed in the primary leaves of BR3 and TE97 cowpea genotypes infected with *Colletotrichum gloeosporioides* at 8 d after infection. *a*, Left: BR3; right: TE97. *b*, Left: TE97; right: BR3. Necrotic symptom development observed in the primary leaves (*c*, *d*) and stems (*e*, *f*) of BR3 cowpea genotype (compatible interaction) at 21 d after infection. In all pictures, two 25-μL drops of 4.10×10^5 conidia mL^{-1} were deposited at each side of the primary leaf main vein and brushed over the entire adaxial leaf surface.

(ITS1, 5.8S, and ITS2) was determined and deposited in the GenBank (accession no. DQ868520). The lengths of ITS1 and ITS2 were 167 and 157 bp, respectively. A BLAST search revealed that the whole sequence (ITS1 + 5.8S + ITS2) had an identity of 99% or greater with ITS sequences from different isolates of *Colletotrichum gloeosporioides*, and all matching sequences were from this same species.

The infection process of the *C. gloeosporioides* isolate was analyzed in the primary leaves of BR3 (susceptible) and TE97 (resistant) cowpea genotypes by light microscopy. After

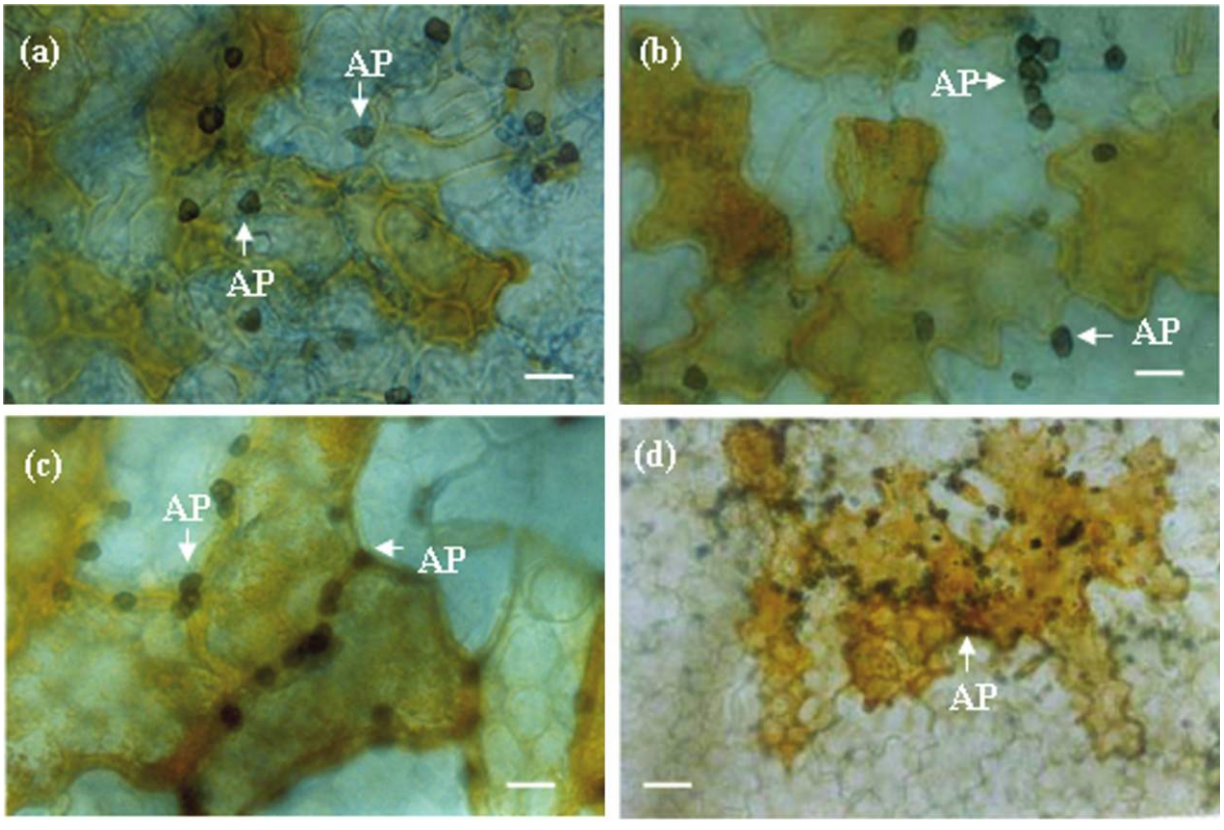

Fig. 6 Micrographs showing necrotic lesions in the primary leaves of BR3 cowpea genotype infected with *Colletotrichum gloeosporioides*. Several appressoria (*AP*) are visible in the necrotic regions. *a–d*, Necroses spreading through several cells at 5, 11, 16, and 22 d after infection, respectively. Staining was done with aniline lactophenol blue. Scale bars = 20 μm for *a–c* and 40 μm for *d*.

inoculation of cowpea primary leaves with *C. gloeosporioides*, conidia produced a septum across the middle of the spore and generated a germ tube at the end of which a melanized appressorium differentiated within 24 HAI (fig. 1*b*). A great number of appressoria were observed both in BR3 (fig. 1) and TE97 (figure not shown) 1 d after inoculation and for several days after. In BR3, however, it was observed that some germ tubes ramified, generating multiple appressoria. Hyphal anastomosis was also noticed after 24 HAI (fig. 1*c*, 1*d*).

In both cowpea genotypes, adhesion of *C. gloeosporioides* spores and appressoria to the adaxial surface of cowpea primary leaves occurred preferentially above the spaces between epidermal cells (fig. 2*a*, 2*b*), and fungal penetration occurred throughout the epidermal cells, typical of a direct process. However, in TE97, penetration through stomata, characterizing an indirect process (fig. 2*c*), was also observed more often than in BR3. The presence of an internal light spot visible in the melanized appressorium within 24 HAI in both genotypes indicated that the appressorium produced a penetration pore and peg as represented typically in figure 1*a* for BR3. Furthermore, the fungus had penetrated the cuticle and had begun to grow in the underlying epidermal wall. However, germinated conidia, melanized appressorium, and the presence of penetration pore structure that appressorium has produced were more frequently observed in BR3 than in TE97. Moreover, in both genotypes the penetration peg swelled to form a spherical infection vesicle with a short neck region inside the epidermal cell (fig. 3*a*), which developed bulbous lateral lobes 48 HAI. Subsequently they became multilobed and multiseptate (fig. 3*b*), and large primary hyphae emerged and grew intracellularly (figure not shown), characterizing the biotrophic phase of infection. Beyond 96 HAI, thin intracellular secondary filamentous hyphae grew out from the lateral lobes and colonized adjacent host cells (fig. 4*a*). However, in TE97, there were fewer infection sites per leaf presenting similar fungal development as above, and the area of necrosis was smaller than that of BR3 (fig. 5). Furthermore, in BR3, 16 d after inoculation, aggregation of secondary septate hyphae was also noticed, albeit sparsely, representing the initial formation of an acervulus, showing two visible melanized setae (fig. 4*b*). Thus, in this work, *C. gloeosporioides* apparently succeeded in establishing a more defined compatible interaction with the BR3 genotype.

Indeed, the macroscopic observations were in agreement with the microscopic findings. Accordingly, large water-soaked necrotic lesions on the surface of BR3, characteristic of a cowpea variety susceptible to anthracnose, were present not only at the site of spore inoculation but also spread to numerous cells and tissues distant from the infection site (fig. 5).These included stems (fig. 5*e*, 5*f*), indicating that the fungus grew systemically. These extensive necrotic lesions were also well evident by light microscopy (fig. 6). However, in TE97, only a few small necrotic lesions restricted to the

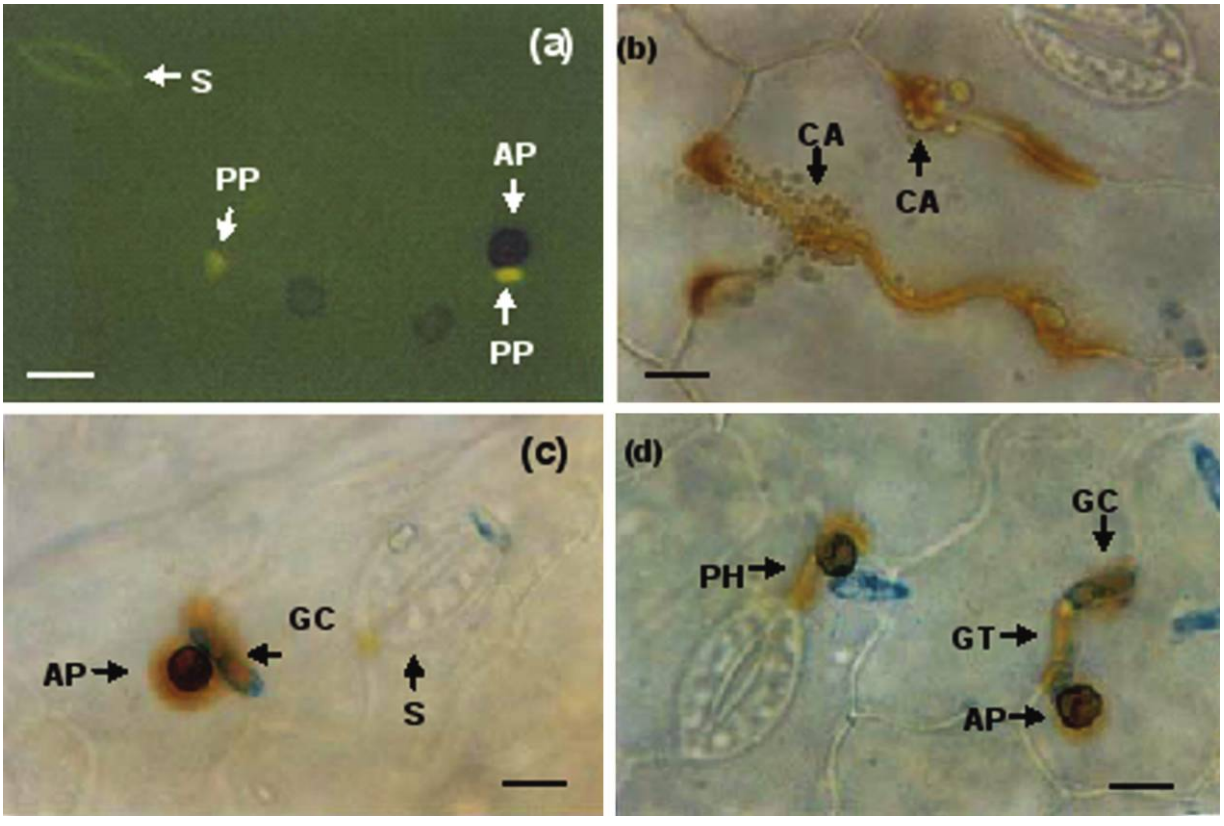

Fig. 7 *a*, Autofluorescent stoma (*S*) and papilla (*PP*) in the primary leaves of TE97 cowpea genotype infected with *Colletotrichum gloeosporioides* at 72 h after inoculation (HAI). Papilla was detected under UV. *b*, Cytoplasmic aggregation (*CA*) in undamaged cells and accumulation of H_2O_2 beneath hyphal structures as seen by accumulation of DAB (3′-3′-diaminobenzidine; reddish brown staining) occurring in the primary leaves of TE97 at 8 d after infection. *c*, *d*, Accumulation of H_2O_2 (reddish brown staining) beneath and/or radial to conidium (*GC*), germ tubes (*GT*), and appressoria (*AP*) of *C. gloeosporioides* in the leaf tissues of TE97 within 48 HAI. Staining was done with aniline lactophenol blue and DAB. Scale bars = 10 μm.

site of infection (fig. 5*a* [right], 5*b* [left]), similar to those observed in incompatible relationships between cowpea and *Colletotrichum* fungi, were visible in the primary leaves. Moreover, although the fungus had rapidly completed its life cycle in TE97, it was restricted to the inoculation site without colonizing neighboring cells or distant tissues. Additionally, light microscopic examination of the inoculated primary leaves in the incompatible interaction between TE97 and *C. gloeosporioides* showed in this cowpea genotype the typical hypersensitive response (HR), papilla formation beneath the appressorium (fig. 7*a*) and cytoplasmic aggregation in undamaged cells adjacent to the necrotic ones (7*b*) in the early stages of fungal infection, within 48 HAI, during the biotrophic phase of the pathogen. Accordingly, in addition to the HR of TE97, papilla formation beneath the appressorium (fig. 7*a*) and cytoplasmic aggregation in undamaged cells adjacent to the necrotic ones (fig. 7*b*) were visible. At the same time, H_2O_2 accumulation, as indicated by reddish brown staining, occurred in the region of papilla formation, beneath and radial to the appressoria, and in the region subjacent to the germ tube (fig. 7*c*, 7*d*), as well as in the epidermal cells undergoing HR (fig. 7*b*). In BR3, callose formation and H_2O_2 accumulation were consistently much less intense and presented lower frequency of HR compared with TE97.

Phenolic compound deposition (fig. 8*a*, 8*b*) and the presence of autofluorescent structures (data not shown) apart from cell wall lignification (fig. 8*c*, 8*d*) were also noted within 72–120 HAI in both TE97 and BR3 genotypes. However, these responses were more prominent for TE97 (fig. 8*a*, 8*c*) in comparison to BR3 (fig. 8*b*, 8*d*). For instance, in agreement with the higher phenolic compound accumulation in TE97, it was noticed that in the inoculated primary leaves of this resistant genotype, PAL presented two phases of higher activity ($P \leq 0.05$) at 12 h and from 24 to 72 HAI (fig. 9), compared with the susceptible genotype BR3. PAL catalyzes the elimination of ammonia from L-phenylalanine to trans-cinnamic acid. Cinnamic acid in turn is the precursor of lignins, flavonoids, and coumarins, which makes PAL a key enzyme in the metabolism of phenylpropanoids in plants.

Discussion

Analysis of the whole nucleotide sequence of the ITS1 + 5.8S + ITS2 regions of rDNA from the *Colletotrichum* isolate of this study showed 99% or greater identity with ITS sequences from different isolates of *Colletotrichum gloeosporioides*. Comparison of the ITS1 and ITS2 sequences

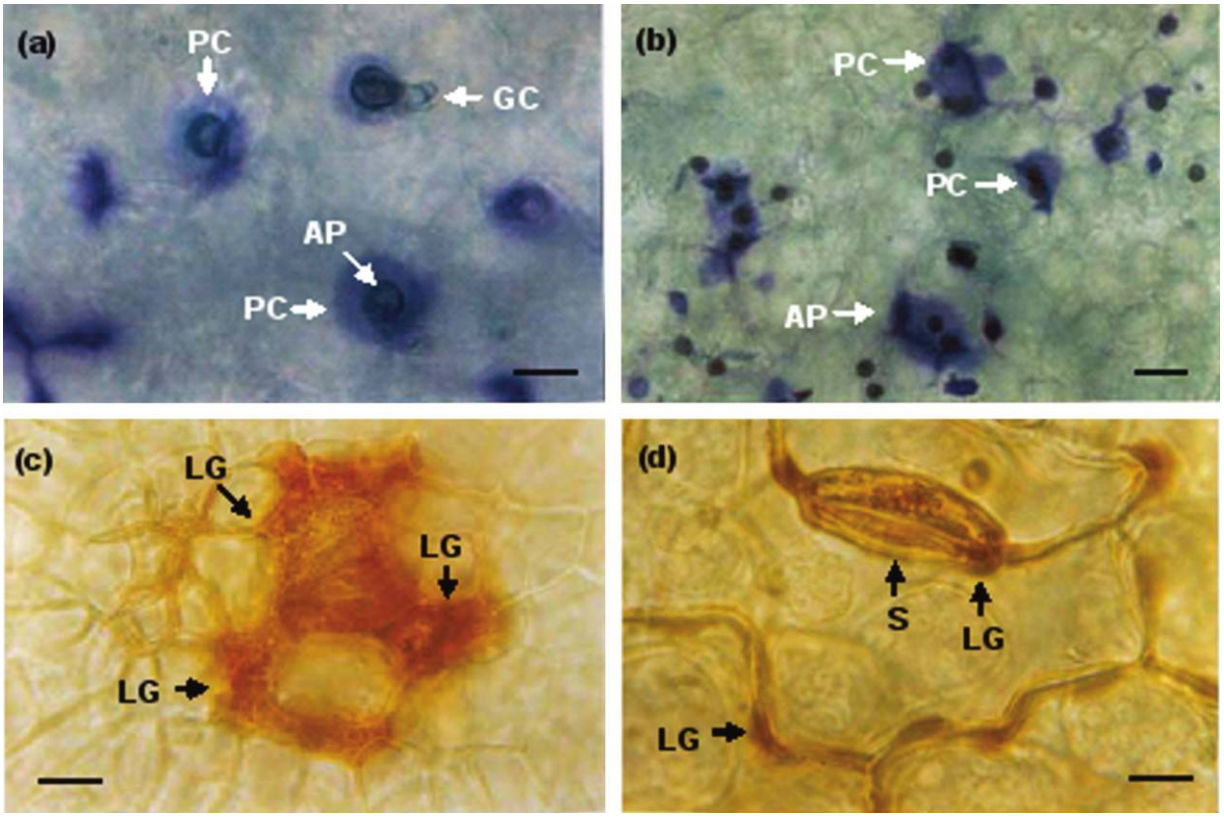

Fig. 8 Deposition of phenolic compounds (PC; blue violet staining circles) beneath and around appressoria (*a*, *b*) and cell wall lignification (brown reddish staining) (*c*, *d*) of primary leaves of TE97 (*a*, *c*) and BR3 (*b*, *d*) genotypes within 5 d after infection with *Colletotrichum gloeosporioides*. Appressorium (*AP*), germinating conidium (*GC*), lignification (*LG*), stoma (*S*). Scale bars = 10 μm for *a*, *c*, *d* and 20 μm for *b*.

(this study) with those from *C. gloeosporioides* f.sp. *aeschynomene* (Nirenberg et al. 2002; GenBank accession no. AJ301986) revealed only two differences, an insertion/deletion in the first spacer (at site 117 in the alignment) and a mutation (T ↔ C) in the second one at position 38. Latunde-Dada et al. (1999) showed that the nucleotide sequences of the amplified D2 and ITS-2 regions of the rDNA from a *Colletotrichum* species causing latent infection and anthracnose in cowpea had 95%–96% identity with isolates of *C. gloeosporioides* from *Aeschynomene virginica*, *Stylosanthes scabra*, and *Mangifera indica*. Thus, on the basis of the nucleotide sequences of ITS1, 5.8S, and ITS2 regions of rRNA, the *Colletotrichum* isolate of this study belongs to the species *C. gloeosporioides* with the ability to cause anthracnose in cowpea.

It was shown here that this isolate of *C. gloeosporioides* penetrated the cuticle and epidermal cell wall of both TE97 and BR3 genotypes by means of a narrow penetration peg that emerged from the appressorium base. This mode of penetration is common among various *Colletotrichum* species (Wharton and Uribeondo 2004) in which differentiation of the appressorium is essential for penetration of the plant cuticle and cell wall and involves a combination of mechanical force, in the form of high turgor pressure, and enzymatic degradation (O'Connell et al. 2000; Latunde-Dada 2001). Penetration of the leaf tissues was also achieved through stomatal openings, although less frequently, by means of undifferentiated germ tubes (fig. 2*c*). This rare indirect mode of penetration was reported to occur in cowpea, cv. IT82E-60, by a *Colletotrichum* species causing latent anthracnose (Latunde-Dada et al. 1999).

The necrotic lesions that developed on BR3 were numerous and reached cells distant from the infection site, indicating that the fungus succeeds in colonizing this cowpea genotype. In contrast, in TE97, although the fungus had rapidly completed its life cycle, it was restricted to the infection site without colonizing neighboring cells. The macroscopic examination of the whole plantlet (fig. 5*a*, 5*b*) was in agreement with the observation above. Indeed, only few small diameter necrotic lesions, restricted to the site of infection, were visible in the primary leaves of TE97. They did not enlarge and were unable to spread to reach neighboring or distant cells and tissues until the end of the experimental period, at 21 d after infection. This could be a localized defense response that prevents further fungal spread.

Typical anthracnose lesions (tan to brown, shrunken, and lenticular) on susceptible varieties enlarge rapidly and coalesce to girdle stems, peduncles, and petioles. In contrast, lesions on resistant varieties are tiny, necroctic flecks or lenticular, and shiny reddish brown (Emechebe and Florini 1997). In this study, the necrotic spots on TE97 restricted to the sites of inoculation in the cowpea primary leaves (fig. 5*a*,

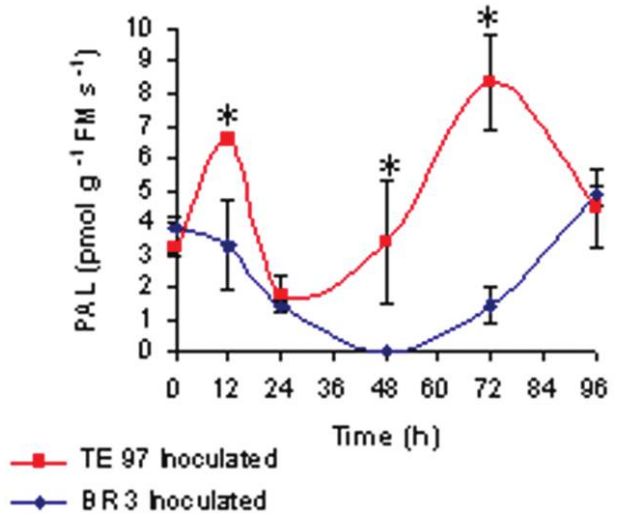

Fig. 9 Time course of PAL activity in the primary leaves of BR3 and TE97 cowpea genotypes inoculated with *Colletotrichum gloeosporioides*. Bars represent standard deviation, and values are compared at each time point. Asterisks show significant differences ($P < 0.05$) according to Student's *t*-test.

5*b*) might be associated with an HR. This response might result from the generation of reactive oxygen species. Indeed, H_2O_2 accumulation was evident in the regions of papilla (callose) formation, beneath the appressorium, and in the regions subjacent to the germ tubes and radial to some cells (fig. 7*b*–7*d*).

In addition to HR and papilla formation beneath the appressorium in TE97 (fig. 7*a*), cytoplasmic aggregation in undamaged cells adjacent to the necrotic ones (fig. 7*b*) was visible. Cytoplasmic aggregation may have resulted from the rearrangement of fine actin filaments at the potential infection sites, as reported for *Nicotiana benthamiana* leaf epidermal cells inoculated with *C. destructivum* and *C. graminicola* (Shan and Goodwin 2005). Strong H_2O_2 accumulation was found in effective papillae and associated cytosolic vesicles in both susceptible and resistant wheat lines to *Blumeria graminis* (*Erysiphe graminis* f.sp. *tritici*), indicating the important role of H_2O_2 in effective papillae formed as a general plant defense against powdery mildew (Li et al. 2005). For instance, in barley attacked by the powdery mildew fungus (*B. graminis* f.sp. *hordei*), H_2O_2 accumulation was not detected in noneffective papillae of cells that had been successfully penetrated (Hückelhoven et al. 1999).

In this study, it was also observed that the presence of autofluorescent papillae (callose) beneath the appressoria in the primary leaves was more frequent in the resistant genotype TE97 (Fig. 7*a*) than in the susceptible BR3. Callose deposition and autofluorescent structures were not observed in control plants. Callose deposition and autofluorescence are markers associated with lesion formation during HR (Lee and Hwang 2005). Generally, papilla formation takes place during fungal infection and is a widely recognized early response of host plants to microbial attack. It has an important role in preventing fungal penetration in the host cells (Thordal-Christensen et al. 1997; Hückelhoven et al. 1999). Thus, in this study, it is assumed that the higher H_2O_2 accumulation and papilla formation in the resistant genotype, TE97, at the early stages of fungal development, during the biotrophic phase of *C. gloeosporioides*, are very important defense responses to this incompatible reaction. Probably early H_2O_2 accumulation functions both as an antibiotic agent and as a signal to trigger a wide array of defense-related genes (Lee and Hwang 2005), which leads to HR and other defense responses.

Antifungal activity of H_2O_2 was seen in our laboratory. Accordingly, 5 mM and 25 mM H_2O_2 concentrations were able to inhibit germination and mycelial growth, respectively, of *C. gloeosporioides* (figure not shown). Thus, unsuccessful colonization and spread of *C. gloeosporioides* in TE97 tissues may have also resulted from the inability of the fungus to prevent local H_2O_2 accumulation, supporting the hypothesis that H_2O_2 may play a crucial role in cowpea defense against *C. gloeosporioides*. For instance, it was reported that the resistance of the white barley mutant albostrians to the biotrophic fungus *B. graminis* f.sp. *hordei* was enhanced in the white leaves, probably as a result of H_2O_2 accumulation in the leaf sites where conidia were germinating (germinating hyphae + appressorium) and in the region of papilla in addition to the expression of various defense genes (Jain et al. 2004).

Several researchers agree with the fact that phenolic compound accumulation precedes both lignification and programmed cell death and constitutes one of the first defense responses of resistant plants (Cohen et al. 1990). Chen and Heath (1994) reported HR in cowpea in response to the rust fungus *Uromyces vignae*. Further, they showed that exogenous reactive oxygen species scavengers, such as the enzymes superoxide dismutase and catalase, diminished the frequency of epidermal cells displaying autofluorescence, 24 h after fungal inoculation (Chen and Heath 1994). In this study, phenolic compounds accumulated in the primary leaves of both cowpea genotypes, BR3 and TE97, when inoculated with *C. gloeosporioides* (fig. 8*a*, 8*b*). The activity of PAL, the first enzyme of the biosynthesis pathway of phenolic compounds, was assessed. In the resistant genotype, TE97, a two-phase kinetic was observed for PAL activity (fig. 9). A rapid and transient increase of PAL activity within 12 HAI and a second prolonged increase in PAL activity between 48 and 72 HAI over that of BR3 were noticed. Phase 1 might be associated with the higher phenolic compound accumulation (fig. 8*a*, 8*b*) at the early stage (within 48 HAI) of attempted penetration and the higher number of autofluorescence structures present in cowpea primary leaves of TE97 compared with BR3 (fig. 7*a*). Recently, Adandonon et al. (2004) reported higher phenol contents in cowpea-tolerant cultivars infected with *Sclerotium rolfsii* compared with the susceptible one. They observed that the highest increase in phenol content was found from 48 HAI, and the net effect of inoculation was a 630% increase in soluble + insoluble phenolics in the stems of tolerant cultivars in comparison with the contents of susceptible ones, wherein increases averaged only 212%.

In our study the increase in PAL activity in phase 2 was probably associated with the biosynthesis of phenolic compounds that accumulated preceding lignification to reinforce the host cell wall (Cohen et al. 1990) against pathogen

penetration. Similarly, a two-phase kinetic was observed for the PAL activity in the leaves of resistant coffee plants (*Coffea arabica* and *Coffea congensis*) at 48 and 120 HAI, associated, respectively, with phenolic compound accumulation and lignification response, which arrested the growth of the orange rust fungus (*Hemileia vastatrix*) (Silva et al. 2002). These authors also reported the presence of autofluorescence at the stomatal guard cells and at subsidiary cells. We observed this in our study (fig. 7*a*) and, additionally, in the mesophyll (as an indicator of resistance). Enhanced resistance to the necrotrophic fungus *Cercospora nicotianae* and to the oomycete *Phytophthora parasitica* f. *nicotianae* was associated with the overexpression of PAL activity in transgenic tobacco (Way et al. 2002).

In the incompatible interaction between soybean (*Glycine max*) and *Phytophthora sojae*, HR, phenolic compound accumulation, lignification, and glyceolin (phytoalexin) biosynthesis were observed. In contrast, for the susceptible variety (cv. Harosoy) these responses were less prominent (Mohr and Cahill 2001). In this work, the red coloration developed around the cell walls of the resistant genotype TE97 after treating its cleared primary leaves with fluoroglucinol-HCl and after inoculation with the fungus *Colletotrichum gloeosporioides*. This suggests a marked deposition of lignin in the epidermal cells (fig. 8*c*). In contrast, this response was minimal (fig. 8*d*) in the susceptible genotype BR3.

In conclusion, it is suggested that resistance of the cowpea genotype TE97 to the hemibiotrophic fungus *C. gloeosporioides* is correlated, at least partly, to HR reaction, higher early H_2O_2 accumulation, enhanced PAL activity with the consequent increase in phenolic compound deposition, enhanced cell wall lignification, and callose (papilla) formation during the early stages of infection.

Acknowledgments

This research was supported by the Council for Advanced Professional Training (CAPES), the National Council for Scientific and Technological Development (CNPq), the Scientific and Technological Development Support Programme (PADCT), and the Program for Academic Cooperation (PROCAD). Ana L. H. Barreto was supported by a doctoral studentship from CAPES, and T. E. Matos and Y. R. G. Eloy received support from the Brazilian Institutional Program for Scientific Initiation (PIBIC)–CNPq.

Literature Cited

Adandonon A, TAS Aveling, T Regnier 2004 Phenolic content in cowpea as a screening parameter for resistance or susceptibility to damping-off and stem rot caused by *Sclerotium rolfsii*. PhD thesis. University of Pretoria.

Altschul SF, W Gish, W Miller, EW Myers, DJ Lipman 1990 Basic local alignment search tool. J Mol Biol 215:403–410.

Bailey JA, RJ O'Connell, RJ Pring, C Nash 1992 Infection strategies of *Colletotrichum* species. Pages 186–202 *in* JA Bailey, MJ Jeger, eds. *Colletotrichum*: biology, pathology and control. CAB International, Wallingford.

Balows A, WJ Hausler, KL Herrman 1991 Manual of clinical microbiology. 5th ed. American Society of Microbiologists, Washington, DC. 1364 pp.

Borden S, VJ Higgins 2002 Hydrogen peroxide plays a critical role in the defence reponse of tomato to *Cladosporium fulvum*. Physiol Mol Plant Pathol 61:227–236.

Bradford MM 1976 A rapid and sensitive method for the quantitation of micrograms quantities for proteins utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Chen CY, MC Heath 1994 Features of the rapid cell death induced in cowpea by the monokarion of the cowpea rust fungus or the monokaryon-derived cultivar-specific elicitor of necrosis. Physiol Mol Plant Pathol 44:157–170.

Cohen Y, H Eyal, J Hanania 1990 Ultrastructure, autofluorescence, callose deposition and lignification in susceptible and resistant muskmelon leaves infected with the powdery mildew fungus *Sphaerotheca fuliginea*. Physiol Mol Plant Pathol 36:191–204.

El-Shora HM 2002 Properties of phenylalanine ammonia-lyase from marrow cotyledons. Plant Sci 162:1–7.

Emechebe AM 1981 Brown blotch of cowpea in northern Nigeria. Samaru J Agric Res 1:20–26.

Emechebe AM, DA Florini 1997 Shoot and pod diseases of cowpea induced by fungi and bacteria. Pages 176–192 *in* BB Singh, DR Mohan Raj, KE Dashiell, LEN Jackai, eds. Advances in cowpea research. International Institute of Tropical Agriculture, Ibadan, Nigeria.

Hoagland DR, DI Arnon 1950 The water culture method for growing plants without soil. Calif Agric Exp Stn Circ 347.

Hückelhoven R, J Fodor, C Preis, K-H Kogel 1999 Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. Plant Physiol 119:1251–1260.

Jain SK, G Langen, W Hess, T Börner, R Hückelhoven, K-H Kogel 2004 The white barley mutant Albostrians shows enhanced resistance to the biotrophic *Blumeria graminis* f. sp. *hordei*. Mol Plant Microbe Interact 17:374–382.

Latunde-Dada AO 2001 *Colletotrichum*: tales of forcible entry, stealth, transient confinement and breakout. Mol Plant Pathol 2: 187–198.

Latunde-Dada AO, JA Bailey, JA Lucas 1997 Infection process of *Colletotrichum destructivum* O'Gara from lucerne (*Medicago sativa*). Eur J Plant Pathol 103:35–41.

Latunde-Dada AO, RJ O'Connell, C Nash, JA Lucas 1999 Stomatal penetration of cowpea (*Vigna unguiculata*) leaves by a *Colletotrichum* species causing latent anthracnose. Plant Pathol 48: 777–784.

Latunde-Dada AO, RJ O'Connell, C Nash, RJ Pring, JA Lucas, JA Bailey 1996 Infection process and identity of the hemibiotrophic anthracnose fungus (*Colletotrichum destructivum* O'Gara) from cowpea (*Vigna unguiculata* (L.) Walp.). Mycol Res 100:1133–1141.

Lee SC, BK Hwang 2005 Induction of some defense-related genes and oxidative burst is required for the establishment of systemic acquired resistance in *Capsicum annuum*. Planta 221:790–800.

Li AL, ML Wang, RH Zhou, XY Kong, NX Huo, WS Wang, JZ Jia 2005 Comparative analysis of early H_2O_2 accumulation in compatible and incompatible wheat–powdery mildew interactions. Plant Pathol 54:308–316.

Maia FMM, JTA Oliveira, MRT Matos, RA Moreira, IM Vasconcelos 2000 Proximate composition, amino acid content and hemagglutinating and trypsin inhibiting activities of some Brazilian *Vigna unguiculata* (L) Walp cultivars. J Sci Food Agric 80:453–458.

Manners JM, S-A Stephenson, C He, DJ Maclean 2000 Gene transfer and expression in *Colletotrichum gloeosporioides* causing antracnose on *Stylosanthes*. Pages 180–194 *in* D Prusky, S Freeman, MB Dickman, eds. *Colletotrichum*: host specificity, pathology and host-pathogen interaction. APS, St. Paul, MN.

Mlícková K, L Luhová, A Lebeda, B Mieslerová, P Pec 2004 Reactive oxygen species generation and peroxidase activity during *Oidium neolycopersici* infection on *Lycopersicon* species. Plant Physiol Biochem 42:753–761.

Mohr PG, DM Cahill 2001 Relative roles of glyceollin, lignin and the hypersensitive response and the influence of ABA in compatible and incompatible interactions of soybeans with *Phytophthora sojae*. Physiol Mol Plant Pathol 58:31–41.

Mori T, M Sakurai, M Sakuta 2001 Effects of conditioned medium on activities of PAL, CHS, DAHP syntase (DS-Co and DS-Mn) and anthocyanin production in suspension cultures of *Fragaria ananassa*. Plant Sci 160:355–360.

Nirenberg HI, U Feiler, G Hagendorn 2002 Description of *Colletotrichum lupini* comb. nov. in modern terms. Mycologia 94: 307–320.

O'Connell R, S Perfect, B Hughes, R Carzaniga, J Bailey, J Green 2000 Dissecting the cell biology of *Colletotrichum* infection processes. Pages 57–77 *in* D Prusky, S Freeman, MB Dickman, eds. *Colletotrichum*: host specificity, pathology and host-pathogen interaction. APS, St. Paul, MN.

Pring RJ, C Nash, M Zakaria, JA Bailey 1995 Infection process and host range of *Colletotrichum capsici*. Physiol Mol Plant Pathol 46: 137–152.

Sambrook J, EF Fritsch, T Maniatis 1989 Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Shan XC, PH Goodwin 2005 Reorganization of filamentous actin in *Nicotiana benthamiana* leaf epidermal cells inoculated with *Colletotrichum destructivum* and *Colletotrichum graminicola*. Int J Plant Sci 166:31–39.

Silva MC, M Nicole, L Guerra-Guimarães, CJ Rodrigues Jr 2002 Hypersensitive cell death and post-haustorial defence responses arrest the orange rust (*Hemileia vastatrix*) growth in resistant coffee leaves. Physiol Mol Plant Pathol 60:169–183.

Silveira JAG, RCL Costa, JTA Oliveira 2001 Drought-induced effects and recovery of nitrate assimilation and nodule activity in cowpea plants inoculated with *Bradyrhizobium* spp. under moderate nitrate level. Braz J Microbiol 32:187–194.

Singh BB, JD Ehlers, B Sharma, FR Freire-Filho 2002 Recent progress in cowpea breeding. Pages 22–40 *in* CA Fatokun, SA Tarawali, BB Singh, PM Kormawa, M Tamo, eds. Challenges and opportunities for enhancing sustainable cowpea production. International Institute of Tropical Agriculture, Ibadan, Nigeria.

Thordal-Christensen H, Z Zhang, Y Wei, DB Collinge 1997 Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. Plant J 11:1187–1194.

Warner SAJ 1996 Genomic DNA isolation and lambda library construction. Pages 51–73 *in* GD Foster, D Twell, eds. Plant gene isolation: principles and practice. Wiley, West Sussex.

Way HM, K Kazan, N Mitter, KC Goulter, RG Birch, JM Manners 2002 Constitutive expression of a phenylalanine ammonia-lyase gene from *Stylosanthes humilis* in transgenic tobacco leads to enhanced disease resistance but impaired plant growth. Physiol Mol Plant Pathol 60:275–282.

Wei YD, KN Byer, PH Goodwin 1997 Hemibiotrophic infection of round-leaved mallow by *Colletotrichum gloeosporioides* f. sp. *malvae* in relation to leaf senescence and reducing reagents. Mycol Res 101:357–364.

Wharton PS, JD Uribeondo 2004 The biology of *Colletotrichum acutatum*. An Jard Bot Madrid 61:3–22.