HOST DEFENSE RESPONSES RESTRICTS THE GROWTH OF THE FUNGUS *Colletotrichum lindemuthianum* IN COWPEA, TE 97 411-1E RESISTANT GENOTYPE

A. L. H. BARRETO[1] , Y. R. G. ELOY[2], T.E. de MATOS[2], A. S. MARTINS-MIRANDA[2], F. C. O. FREIRE[3], F.R. FREIRE FILHO[1], I. M. VASCONCELOS[2] e J. T. A. OLIVEIRA[2]

Abstract – Cowpea is the main subsistence crop in the semiarid North-east of Brazil. However, very few is known concerning to its defense mechanisms against fungi. The objective of this present work was to evaluate the infection strategies of the hemibiotrophic fungi *Colletotrichum lindemuthianum* and the associated cytological defense responses of two cowpea genotypes, TE 97-411-1E (resistant) and BR 3 Tracuateua (susceptible). The infection process of the fungi in both genotypes occurred preferentially through the leaf epidermal cells by penetration tubes emerged from appressoria. The resistant cowpea genotype, TE 97-411-1E, showed enhanced penetration resistance to *C. lindemuthianum* associated with higher epidermal H_2O_2 accumulation, papilla formation, and increase in 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 TE 97-411-1E cell death was also observed but only in a reduced percentage of the infection sites. In summary the results obtained in the present study suggested that TE 97-411-1E genotype is more resistant to *C. lindemuthianum* compared to BR 3 Tracuateua, as it developed more effective defense responses against the establishment of the pathogen.

Keywords: *Vigna unguiculata*, *Colletotricum lindemuthianum*, infection process.

RESPOSTAS DE DEFESA IMPEDEM O CRESCIMENTO DO FUNGO *Colletotrichum lindemuthianum* NO GENÓTIPO RESISTENTE DE FEIJÃO-DE-CORDA, TE 97 411-1E

Resumo – O feijão-caupi é a principal cultura de subsistência no semi-árido do Nordeste do Brasil. Entretanto, muito pouco é conhecido sobre seu mecanismo de defesa contra fungos. O objetivo do presente trabalho foi avaliar as estratégias de infecção do fungo hemibiotrófico *Colletotrichum lindemuthianum* e as respostas de defesa dos dois genótipos de feijão-caupi, TE 97-411-1E (resistente) e BR 3 Tracuateua (suscetível). O processo de infecção dos genótipos ocorreu, preferencialmente, nas células epidérmicas das folhas por meio de tubos de penetração que emergiram de apressórios do fungo. O genótipo TE 97-411-1E mostrou maior resistência à penetração para *C. lindemuthianum* associado com alto acúmulo de H_2O_2, formação de papila, e aumento na atividade da fenilalanina amônia liase, possivelmente relacionado ao acúmulo de compostos fenólicos e à lignificação da parede celular do hospedeiro. Exame macroscópico das folhas primárias revelou a presença de lesões necróticas profundas, características de antracnose em BR 3 Tracuateua infectada, enquanto que em TE 97-411-1E

[1]Embrapa Meio-Norte,Av. Duque de Caxias, 5650, Bairro Buenos Aires, CEP 64006-220, Teresina, PI. E-mail: analucia@cpamn.embrapa.br
[2]Universidade Federal do Ceará, Departamento de Bioquímica e Biologia Molecular, Fortaleza, CE. E-mail: jtaolive@ufc.br
[3]Embrapa Agroindústria Tropical, Fortaleza, CE.

morte celular foi também observada mas em porcentagem reduzida dos sítios de infecção. Em resumo, os resultados obtidos no presente trabalho mostram que o genótipo TE 97 411-1E é mais resistente ao *C. lindemuthianum* comparado com BR 3 Tracuateua devido este ter desenvolvido mais respostas efetivas de defesa contra o estabelecimento do patógeno.

Palavras chave: Feijão-caupi, *Colletotrichum lindemuthianum*, processo de infecção.

Introduction

Cowpea is an important food legume which provides calories, proteins, minerals and vitamins in the semiarid Northeast of Brazil (Emechebe & Florini, 1997). Unfortunately cowpea is susceptible to a wide range of pathogens which attack all plant parts during its life cycle. These include viruses, bacteria, fungi, nematodes, and insects which restrain production, particularly grain yield. Fungi are amongst the most serious pathogens that infect cowpea. In the group of *Colletotrichum* various species, including *C. lindemuthianum*, *C. gloeosporioides* are considered major cowpea pathogens causing important economic losses. *Colletotrichum* species use different strategies to infect their hosts 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 a combination of both. The fungus has an initial biotrophic phase where it feeds on living host cells and the host is symptomless. This is followed by a destructive necrotrophic phase when the fungus causes extensive degradation of host cells and symptoms become visible (Bailey *et al*., 1992).Thus in view of the importance of *C. lindemuthianum* as a major fungal pathogen of cowpea and the need to understand the mode of infection of individual *Colletotrichum* species for developing effective control strategies, particularly those based on host plant resistance, the current study was undertaken. The aims were to investigate the infection process on cowpea leaves in compatible and incompatible interactions and, in addition, to assess immediate defense responses performed by the host.

Material and Methods

The fungus was cultured on potato dextrose agar (PDA). Conidia were obtained from 12-day-old. Seeds of TE 97-411-1E and BR 3 Tracuateua cowpea genotypes were obtained from Embrapa Meio-Norte. Seeds were disinfected with 0.05% active chloride and sown in autoclaved river sand. They were kept in a greenhouse and irrigated with water for up to 4 days after sowing and subsequently with diluted Hoagland & Arnon (1950) solution. Ten days after sowing healthy plantlets were transferred to a growth chamber kept at 25-30 °C, 85 ± 5% relative humidity with a 12 h photoperiod. Two days later, the primary leaves were inoculated by applying two 25 mL equidistant droplets of spore suspension (4×10^5 mL^{-1} in sterile distilled water) at 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 hours after inoculation (hai) to analyze phenylalanine ammonia-lyase activity and at 1, 2, 3, 4, 5, 6, 8, 11, 16, and 22 days after infection (dai) to carry out the microscopic analyses.

Detection of autofluorescence was performed using the method described by Borden & Higgins (2002). Phenolics were visualized by staining with toluidine blue as violet-blue structures. To detect callose (papillae) formation, the leaf pieces were treated with phosphate buffer and stained with a buffered solution of aniline blue. For lignin localization decolorized leaf pieces were immersed in phloroglucinol-HCl reagent (Borden & Higgins, 2002**).** To detect H_2O_2 accumulation leaves were infiltrated with 3'-3'-diaminobenzidine (DAB) (Thordal-Christensen *et al*., 1997).

PAL (EC 4.3.1.5) activity was measured according the method described by El-Shora (2002).

Results and Discussion

Following deposition on the leaf surface, conidia germinated and formed apressoria within 24 h of inoculation (Figure1a). In both genotypes, the pathogen gains ingress into the host by elaborating from the melanized apressorium an infection peg, which penetrates the cuticle directly to initiate infection, typical of a direct process. However, in TE97, penetration through plants stomata, characterizing an indirect process, (Figure1b), was more frequently observed in TE 97 than in BR3. Moreover a great number of germinated conidia, melanized appressorium and the presence of penetration pore structure were more numerous in BR3 genotype than in TE97. In both genotypes the penetration peg swelled to form a spherical infection vesicle with a short neck region inside the epidermal cell (Figure 1c), which developed bulbous lateral lobes 48 h after inoculation. Subsequently, they became multilobed and a multiseptated (Figure 1d) and large primary hyphae emerged and grew intracellularly (Figure not shown) characterizing the biotrophic phase of infection. Beyond 4 dai, thin intracellular secondary filamentous hyphae grew out from the lateral lobes (Latunde-Dada et al, 1999) and colonized adjacent host cells (Figure 1e). Furthermore, in BR3, 16 dai aggregation of secondary septated hyphae was also noticed, albeit sparsely, representing the initial formation of an acervulus (Figure 1f) as reported in cowpea cv. IT82E-60 leaves infected with a lucerne isolate of *C. destructivum* 7 dai (Latunde-Dada et al., 1999). Thus in this present study *C. lindemuthianum* apparently succeeded in establishing a more defined compatible interaction with BR3 genotype. Indeed, the macroscopic observations were in agreement with the microscopic findings. Accordingly, large water-soaked necrotic lesions on the surface of BR3 primary leaves, characteristic of a susceptible cowpea variety to anthracnose (Emechebe & Florini, 1997), were present not only at the site of spore inoculation but spreading to numerous cells and tissues distant from the infection site. These extensive necrotic lesions were also well evident by light microscopy (Figure 2a). On the other hand, in TE97, only few small necrotic lesions restricted to the site of infection, similar to those observed in incompatible relationships between cowpea and *Colletotrichum* fungi (Emechebe & Florini, 1997), 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. Accordingly, in addition to the hypersensitive response (HR) of TE97, papilla formation beneath the appressorium (Figure 2b) and cytoplasm aggregation of undamaged cells adjacent to the necrotic ones (Figure 2c) was visible within 2 dai.

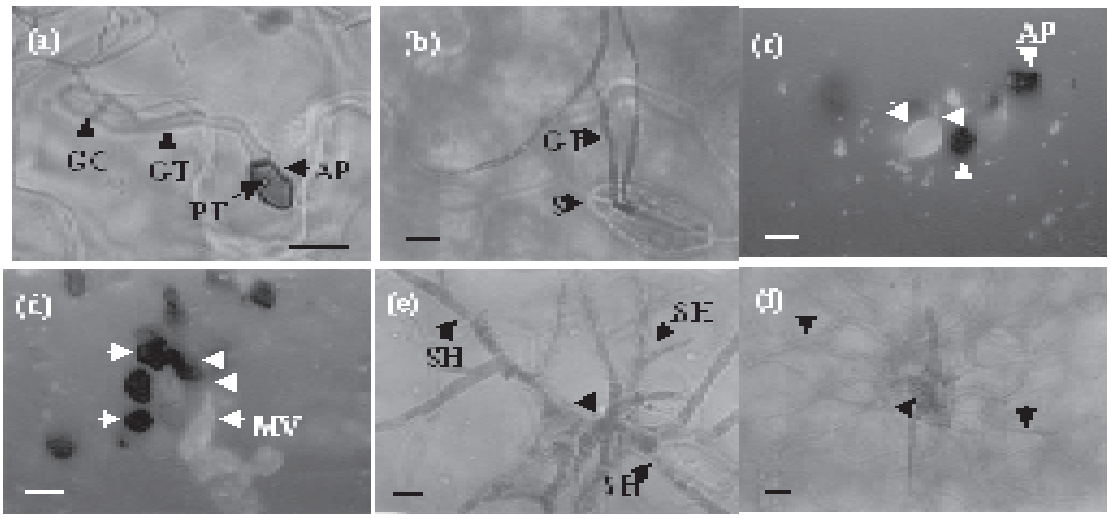

Figure 1. Infection structures of *C. lindemuthianum* in the primary leaves of cowpea genotypes. (a) fungal infection structures in BR3 3 days after inoculation (dai); (b) indirect penetration of *C. lindemuthianum* through stomatum in TE97; (c) Spherical infection vesicle (IV) with a short neck region (arrowhead) emerged from an appressorium in infected BR3; (d) Multilobed vesicle (MV) with lateral lobes in BR3 5 dai; (e) Irradiation of secondary hyphae (SH) from a multilobed vesicle (MV) colonizing adjacent host cells in TE97 4 dai; (f) Irradiation of melanized secondary hyphae (MH) and initial formation of acervulus in BR3 16 dai. Appressorium (AP), germinating conidium(GC), germinating tube(GT),penetration tube(PT). Bars = 10 mm (a),(b),(c),(d),(e); 20 mm (f).

At the same time, H_2O_2 accumulation occurred in the region of papilla formation, beneath and radial to the appressoria and in the region subjacent to the germinating tube (Figure 2d), as well as in the epidermal cells undergoing HR. In BR3, callose formation and H_2O_2 accumulation presented lower frequency of HR compared to TE97. Phenolic compound deposition (Figure 2e) and the presence of autofluorescent structures apart from cell wall lignification (Figure 2f) were more prominent for TE 97 in comparison to BR3 within 3-5 dai.

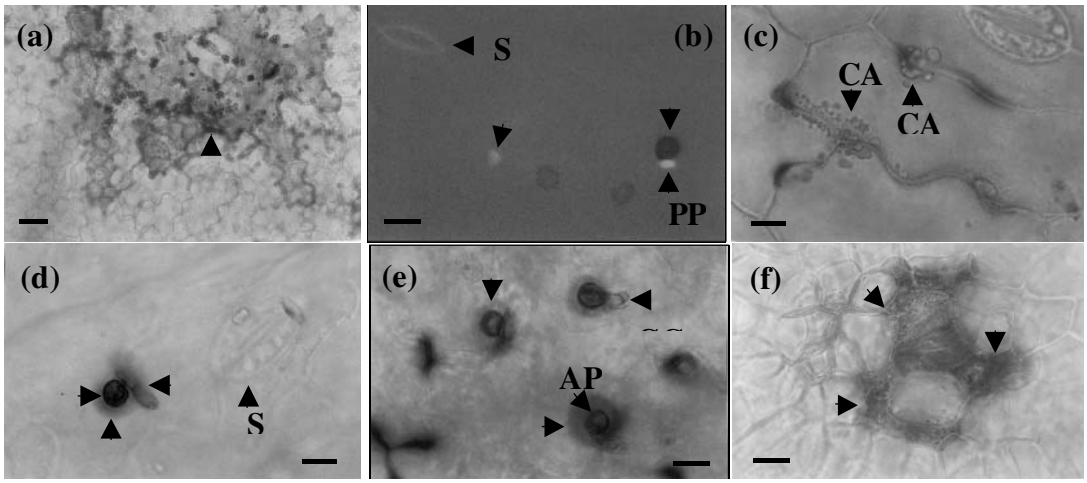

Figure 2. Defense responses of the primary leaves of cowpea genotypes infected with *C. lindemuthianum*. (a) Necrotic lesions in BR3 at 22 dai; (b) Autofluorescent stomatum (S) and pappila (PP) in TE 97 at 3 dai; (c) Cytoplasm aggregation (CA) in TE 97 at 8 dai; (d) Accumulation of H_2O_2 beneath and/or radial to conidium, germinating tubes and appressoria in TE 97 at 2 dai; (e) Deposition of phenolic compounds beneath and around appressoria; (f) and cell wall lignification in TE97 at 5 dai. Bar s = 10 mm (b), (c), (d), (e) and (f); 40 mm (a).

In the inoculated primary leaves of the resistant genotype, TE97, PAL presented two phases of higher activity ($p = 0.05$) at 12 h and from 24 to 72 hai (Figure 3), compared to 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 (Alunni et al., 2003).

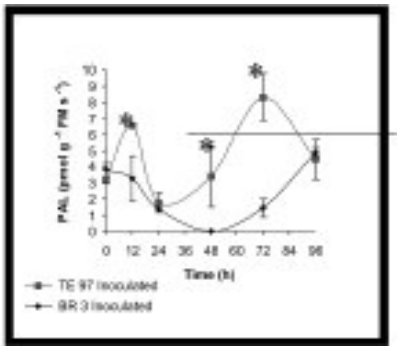

Figure 3. Time course of PAL activity in the primary leaves of BR3 and TE97 cowpea genotypes inoculated with *C. lindemuthianum*. Bars represent standard deviation and values are compared at each time point. Asterisks show significant differences ($r < 0.05$) according to Student's t-test

Cknowledgements

This research was supported by CAPES, CNPq, PADCT, PROCAD. T. E. Matos and Y. R. G. Eloy were supported by PIBIC-CNPq program.

References

ALUNNI S, CIPICIANI A, GIOVANNA-FIORONI G, OTTAVI L. Mechanisms of inhibition of phenylalanine ammonia-lyase by phenol inhibitors and phenol/glycine synergistic inhibitors. **Archives of Biochemistry and Biophysics,** v. 412, 170-175, 2003.

BAILEY J.A, O'CONNELL R.J, PRING R.J, NASH C. Infection strategies of *Colletotrichum* species. In: Bailey J.A., Jeger MJ, eds. ***Colletotrichum*: Biology, Pathology and Control.** Wallingford: CAB International, 1992. p. 186-202.

BORDEN, S., HIGGINS, V. J. Hydrogen peroxide plays a critical role in the defence reponse of tomato to *Cladosporium fulvum*. **Physiological and Molecular Plant Pathology**, v. 61, p. 227-236, 2002.

EL-SHORA, H.M. Properties of Phenylalanine Ammonia-Lyase from Marrow Cotyledons. **Plant Science**, v. 162, p. 1-7, 2002

EMECHEBE A.M., FLORINI D.A. 1997. Shoot and pod diseases of cowpea induced by fungi and bacteria. In: Singh BB, Mohan Raj D.R, Dashiell K.E, Jackai L.E.N. eds. **Advances in Cowpea Research**. Ibadan: International Institute of Tropical Agriculture: Japan International Research Center for Agricultural Sciences. 1997. p.176-192.

HOAGLAND, D. R., ARNON, D. I. The water culture method for growing plants without soil. **California Agriculture Experimental Station of University of California, Berkeley**, v. 347, p. 139, 1950.

LATUNDE-DADA A.O, O'CONNELL R.J, NASH C, LUCAS J.A. Stomatal penetration of cowpea (*Vigna unguiculata*) leaves by a *Colletotrichum* species causing latent anthracnose. **Plant Pathology**, v. 48, p. 777-784, 1999.

THORDAL-CHRISTENSEN, H., ZHANG, Z., WEI, Y., COLLINGE, D. B. Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during barley-powdery mildew interaction. **Plant Journal**, v. 11, p. 1187-1