

Cytological aspects of compatible and incompatible interactions between cashew (*Anacardium occidentale* L.) seedlings and isolates of *Colletotrichum gloeosporioides* (Penz.) complex

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Abstract—A strategy in the control anthracnose of cashew (*Anacardium occidentale* L.) is the management of crop phenology and defense mechanisms of this host. In previous studies, under controlled conditions, the seedling reactions of 5 cashew clones (CAP-14, CCP-06, CCP-09, CCP-76 and CCP-1001) to 36 isolates of *Colletotrichum gloeosporioides* Penz. complex (LARS-905 to 940) was evaluated. However, good field management requires information about the infection process. This research aimed to clarify cytophysiological aspects of three compatible interactions of this pathosystem (isolates LARS-905 and 910 × CCP-76; LARS-910 × CCP-1001) and an incompatible one (LARS-905 × CCP-1001), using infected leaves/stems and microscopy (light, scanning and transmission electron). No significant differences were found prior to penetration. In the susceptible combinations, 36–66 h after inoculation, a thin primary hypha (TPH) formed in the invaded epidermal cell, widening as a large primary hypha (LPH), which filled the cell lumen simultaneously with accumulation of yellow-brown lignopolysaccharides. Then, a thin secondary hypha (TSH) developed from the LPH, penetrating adjacent cells before the first became necrotic. In the incompatible interaction, the response of the first invaded cell was faster and more intense, with formation of papilla and lignopolysaccharide-protein-silicon complex usually blocking the pathogen.

Index terms: anthracnose; defense mechanisms; hemibiotrophic fungus; papilla; resistance.

Aspectos citológicos das interações compatíveis e incompatíveis entre mudas de cajueiro (*Anacardium occidentale* L.) e isolados do complexo *Colletotrichum gloeosporioides* (Penz.)

Resumo - Uma estratégia no controle da antracnose do cajueiro (*Anacardium occidentale* L.) é o manejo da fenologia e mecanismos de defesa deste hospedeiro. Em estudos anteriores, em condições controladas, as reações de mudas de 5 clones de caju (CAP-14, CCP-06, CCP-09, CCP-76 e CCP-1001) a 36 isolados do complexo *Colletotrichum gloeosporioides* Penz. (LARS-905 a 940), foram avaliadas. Contudo, um bom manejo em campo requer informações sobre o processo de infecção. Esta pesquisa visou a avaliar aspectos citofisiológicos de três interações compatíveis deste patossistema (isolados LARS-905 e 910 × CCP-76; LARS-910 × CCP-1001) e uma incompatível (LARS-905 × CCP-1001), utilizando folhas/caules infectados e técnicas de microscopia (luz, varredura e transmissão eletrônica). Nenhuma diferença significativa foi encontrada antes da penetração. Nas combinações suscetíveis, 36–66 h após a inoculação, uma delgada hifa primária (TPH) formou-se na célula epidérmica invadida, alargando-se como uma larga hifa primária (LPH), que preencheu o lúmen da célula, simultaneamente com o acúmulo de lignopolissacarídeos amarelo-acastanhados. Então, uma fina hifa secundária (TSH) desenvolveu-se a partir do LPH, penetrando nas células adjacentes antes que as primeiras se tornassem necróticas. Na interação incompatível, a resposta da primeira célula invadida foi mais rápida e intensa, com papila e complexo lignopolissacárico-proteico-siliconoso geralmente bloqueando o patógeno.

Termos para indexação: antracnose; mecanismos de defesa; fungo hemibiotrófico; papila; resistência.

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Introduction

The first Europeans who arrived in Brazil in the 16th century found a land with exotic plant-fruits, such as cashew trees (*Anacardium occidentale* L.), transporting them by sea to Africa (Mozambique, Angola, Kenya and Madagascar) and Goa (India). In India, cashew gradually gained commercial importance through the consumption of its nuts, becoming known as the “gold mine of wasteland”, since it is a good crop for soil conservation, reforestation and wasteland development (SATAPATHY and BEURA, 2018). Later, in the 20th century, the North American and European acceptance of cashew nuts, boosted its marketing from India to the world. Brazil’s Northeastern semi-arid only entered this international chestnut market in the 1950s, but even more so in the 1970s, mainly in the dry season of the year, after government incentives for the development of cashew nut and juice processing (ALBUQUERQUE; SILVA, 2008).

Despite this, since 2012 the country’s participation in the world cashew market is decreasing, not only because of improved technologies provided by incentives from other governments in the market, but due to the strong and prolonged drought in the Northeast region from 2012 to 2016, associated the occurrence of pests and diseases, such as anthracnose (VIDAL, 2016) caused by members of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. species complex, i.e. *C. chrysophilum*, *C. fragariae*, *C. fructicola*, *C. gloeosporioides sensu stricto*, *C. queenslandicum*, *C. siamense* and *C. tropicale* (COMÉ, 2014; NORSHIE, 2020; VELOSO et al., 2018). This reduced the cashew plantation area by 144,000 hectares, leading producers to eradicate the dead cashew trees and to trade their wood (VIDAL, 2016). In fact, the losses caused by anthracnose to cashew trees were recognized in all producing countries (KHATOON et al., 2017; MONTEIRO et al., 2015), although there was no published research on the cytology, ultrastructure and physiology of the infection process on such a host.

Studies of the disease in compatible interactions of other pathosystems have shown that the type and sequence of early cytological infection events can differ markedly. In the systems bean- *C. lindemuthianum*, alfalfa- *C. destructivum*, pea- *C. truncatum*, ripe blueberry fruit- *C. acutatum* and *Arabidopsis thaliana*-*C. higginsianum*, the initial colonization of susceptible epidermal cells is via intracellular biotrophic hyphae (O’CONNELL; BAILEY, 1988; O’CONNELL et al., 1993; LATUNDE-DADA et al., 1996; WHARTON; SCHILDER, 2008; O’CONNELL et al., 2012). For other species, such as from *C. gloeosporioides* complex, different isolates, although sharing similar morphological characteristics, have distinct modes of penetration and colonisation in different hosts (TEBEEST et al., 1978;

MORIN et al., 1996; ZULFIQAR et al., 1996; WEI et al., 1997; BARRETO et al., 2007; PANDEY et al., 2012), however, once a compatible interaction has been established, the pathogen switches to a very destructive mode of growth, causing extensive cell wall dissolution and cell death.

According to López and Lucas (2010a), after assessing the reaction of one non-commercial (CAP-14) and four commercial dwarf cashew clones (CCP-06, CCP-09, CCP-76 and CCP-1001), in controlled environmental conditions, against 36 isolates of *Colletotrichum*, it was found that all the strains, including those from hosts other than cashew, were able to cause lesions on leaves and stems of most clones, albeit to different degrees. The isolates were obtained from anthracnose lesions of cashew and associated host plants in Brazil, and had been previously identified (by cultural, morphological and partial sequences of their 28S ribosomal DNA characters) as members of the *C. gloeosporioides* complex (López and Lucas, 2010b). While the clone CCP-06 was susceptible to most isolates, the clone CCP-1001 was resistant to many of them, including LARS-905, originally isolated from leaves of a blackberry plant (*Rubus brasiliensis*) in a cashew orchard, while the isolate LARS-910, originally obtained from cashew leaves, was aggressive to all clones. On the other hand, injury increased the susceptibility to all isolates, indicating that resistance may also be associated with structural barriers that hinder penetration.

Based on these results, this research aimed to investigate the infection process of two isolates of the *C. gloeosporioides* complex (LARS-905 and 910) on stems/leaves of seedlings of three cashew clones (CCP-76, CCP-1001 and CAP-14), providing information to improve understanding of the host-pathogen interactions, and aid disease management.

Materials and methods

The leaves and stems of cashew seedlings (60-days-old) of the clone CCP-1001 × LARS-905 (member of *C. gloeosporioides* complex originally isolated from anthracnose lesion of *R. brasiliensis* leaf, in Maceió-AL), an incompatible interaction, as well the ones of compatible interactions of the clones CCP-76, CCP-1001 and CAP-14 × LARS-910 (member of *C. gloeosporioides* complex originally isolated from anthracnose lesion of *A. occidentale* leaf, in Maceió-AL), or CCP-76 and CAP-14 × LARS-905, were investigated by microscopy. Pathogen behaviour on the host surface and the initial penetration process were studied by light and scanning microscopy (LM and SEM). Infection hyphae were visualized in stained semi-thin and ultra-thin sections of resin embedded samples, by LM and transmission electron microscopy (TEM).

The ultrastructural relationship between the intracellular primary hyphae and host cell cytoplasm was followed to determine the effects on host cells, including the host responses.

Inoculation and whole tissue clearing and fixing

The detached leaves or stem segments were inoculated and incubated, as described by López and Lucas (2010a). At intervals after inoculation (12, 18, 24, 30, 38, 48, 72, 96, 114, 132, 158 and 182 h), tissues beneath inoculation droplets of twelve young stem segments (*ca.* 5 cm diameter) and three detached young first leaves of cashew seedlings were sliced with a double-edge razor blade. Five thin longitudinal strips of stem segments (*ca.* 0.5 mm thick) and five leaf discs (*ca.* 4 mm diameter), collected at each interval, were immediately placed in vials containing *ca.* 2ml of clarifying solution [0.15 % (w/v) Trichloroacetic acid (TCA) in a 3:1 mixture of Ethanol: Chloroform (EtOH: CHCl₃)]. Then, these vials were immersed in a water bath (70 °C, *ca.* 1.5 h), until the tissues became clear. The assay was repeated three times, and cleared tissues were also used in histochemical tests.

Fixing, dehydrating and embedding tissue for semi-thin sectioning

Detached leaves or stem segments were inoculated according to López and Lucas (2010a). Strips (*ca.* 3 mm × 1 mm × 0.5 mm) of the tissue beneath the inoculum droplets, collected at the same times as described above, were immediately placed in vials containing *ca.* 2ml of 2.5 % (v/v) glutaraldehyde in 0.05M phosphate buffer (Pb), pH 7.2, for infiltration under rotation (2h, including 10 min of vacuum infiltration). Then, the strips were rinsed in 0.05M Pb (pH 7.2) for 3min, in sterilized deionized water (SDW) for 3 min, and dehydrated under rotation (5 h), through an EtOH series [25% (30 min), 50% (30 min), 70% (60 min), 90% (60 min) and 100% EtOH dehydrated over molecular sieve (60 min, 2 changes)]. So, the material was gradually infiltrated with an LR White resin (London Resin, Agar Scientific, Essex, UK) series, added to the vials under rotation [+ 0.5 ml resin (30 min), + 1ml (30 min), + 2 ml (60 min), + 4 ml (60 min)], transferred to vials with 100% resin (60 min, but left open for EtOH evaporation), and at 24 h intervals, for 3 days, the resin was replaced. Finally, the strips were polymerised in flat embedding moulds (oven at 65 °C, nitrogen replacing oxygen) in a fume cupboard. Semi-thin sections (1-2 µm) of resin-embedded tissue were cut with either a LKB microtome or a Reichert-Jung ultramicrotome.

Bright field and fluorescence - light microscopy (LM)

After a preliminary bright field observation of the mounted sections (Zeiss MC63 microscope), samples were viewed with a Zeiss Axiophot microscope equipped with Nomarski differential interference contrast (NDIC) optics and/or UV - epifluorescence. Photomicrographs were recorded [Kodak Technical Pan film (80 ASA) or Kodak TMAX-pro film (400 ASA), when UV- epifluorescence was required, and developed in DIAFINE (Acufine Inc., Chicago, U.S.A.), or Kodak Ektachrome daylight (100 ASA) film], according to the manufacturer's instructions, and then digitized.

Scanning electron microscopy (SEM)

Strips of leaf tissue (*ca.* 6 × 6 mm²) beneath the inoculum drop were cut 48 h after inoculation, and attached to a copper specimen stub using a mixture of colloidal graphite and "Tissue Tec" glue (Agar Scientific, UK). Then, the specimens were frozen by placing the holder on the pre-chamber block (-130 °C) of a Hexland CT 1000 Cryo preparation unit (Oxford Instruments, UK). The pre-chamber was evacuated, and the specimens transferred to the cold microscope stage (SEM chamber) were examined using a Philips 505 SEM (4kV, -180 °C). When ice particles were present, the specimens were discarded, and the procedure was repeated. The tissues were then transferred to the pre-chamber, sputter coated with *ca.* 20 nm of gold, and returned to the microscope cold unit. The tissues were examined using an accelerating voltage of between 7.9kV and 8.1 kV, with the cold stage held at -130 °C to -180 °C. Photographs were taken on Kodak T-Max 100 film and digitized.

Staining of leaf section grids and transmission electron microscopy (TEM)

Ultra-thin sections of the LR-white embedded specimens (obtained as described in 2.2) were cut with a diamond knife using a Reichert-Jung ultramicrotome. They were mounted on Formvar-coated gold slot grids and double-stained with uranyl acetate (UA) followed by lead citrate solution, as described by Reynolds (1963). The grids were floated face down (3 min) on top of UA drops and placed on Parafilm. Just before the end of staining, drops of lead citrate were placed on another Parafilm strip, inside a small Petri dish containing pellets of NaOH to absorb CO₂. The grids were then washed with a jet of fresh DW and immediately floated face down on top of lead citrate drops (*ca.* 2 min) and then dipped in 0.1 N NaOH solution followed by a jet of boiled SDW. Grids were finally blotted dry with filter paper, and examined in a Hitachi H7000 TEM operating at an accelerating voltage of 100 kV.

Analysis of the infection process

The cleared sections (TCA: EtOH: CHCl₃) to study the time course of infection by LM were either examined directly, after mounting in 15-30 µl of aqueous glycerol (30%) on slides under cover slips, or stained with 0.025% (w/v) Aniline blue (Ab) in lactophenol (60 min at room temperature), washed in SDW and then mounted in aqueous glycerol (30%). Also, a KOH-Ab technique of Hood and Shew (1996) was used in some cases, to reveal fungal structures by fluorescence. For this, cleared samples were immersed in 1M KOH for 30 min, rinsed in SDW, mounted in 0.05% (w/v) Ab in 0.067 M K₂HPO₄ (pH 9.0), and examined using excitation filter G365, dichroic mirror FT 396 and barrier filter LP 420.

The percentages of spore germination, appressoria formation and penetration (papilla, large primary and secondary hypha formed), for 100 conidia and appressoria at three different sites of five samples (time and interaction) were recorded. To clarify the mode of penetration and host cell responses, semi-thin sections of resin embedded material were mounted on glass slides at 60 °C and stained (1 min) with a solution of 0.5 % (w/v) Toluidine blue (Tb) in 0.1% (w/v) aqueous Na₂CO₃. These sections were examined by LM, with or without NDIC and photomicrographed (as described to SEM).

Assessment of membrane integrity

The viability of infected cells and the functional integrity of their membranes are affected by fungal colonisation. The of living plant cells plasmolysis reflects the semi-permeable nature of the plasmalemma and tonoplast membranes (OPARKA, 1994), since in a hypertonic salt solution, water is withdrawn from the vacuole by osmosis (the protoplast contracts). Then, strips of whole stem tissues, inoculated and collected as above described, were immersed for 15 min in a fresh solution of 0.85 M KNO₃ in 5 mM Pb (pH 7.5). For the first 5 min, the vials with samples were placed under vacuum to aid infiltration (BAILEY et al., 1990). Treated tissues were mounted on slides, covered with cover slips and observed immediately by bright field and NDIC microscopy to record the percentage of plasmolysed cells. Photomicrographs were made as described to SEM.

Histochemical analysis of whole tissue

Three samples of stem strips and leaf discs were collected 48-120 h after inoculation, cleared (as above) and used in tests for detecting β-1,3-glucans (callose), according to Wilson and Coffey (1980) and also Heath (1987); lignin-like compounds or simple phenols, according to Campbell et al. (1937), Towers and Gibbs (1953), O'Brien et al. (1964), Harris et al. (1982), Herr (1992), Cadena-Gomez and Nicholson (1987) and Bervingson et

al. (1994); condensed tannins and flavonoids, according to Reeve (1959), Jensen (1962) and Harborne (1991); and laccase (*para* or *ortho*-diphenol: O₂ oxidoreductase, E.C.1.10.3.2), peroxidase (donor: H₂O₂ oxidoreductase, E.C.1.11.1.7), tyrosinase (*o*-diphenol: O₂ oxidoreductase, E.C.1.10.3.1) or cytochrome oxidase (O₂ oxidoreductase, E.C.1.9.3.1), according to Harkin and Obst (1973). When the cleared stems and leaves displayed yellow browning granulation that obscured fungal structures, they were or mounted in 0.5-10MNaOH (1-2 min), to disrupt protoplasm and dissolve pigmentation, or immersed in a boiling (100 °C) 1:1 (v/v) mixture of 30% H₂O₂ and concentrated glacial CH₃COOH, for 30-90 min, at intervals of 10 min, for dissolving *o*-quinoid-like compounds, and the infection sites were evaluated by LM.

Finally, samples (stem and leaf tissues) were stained for *ca.* 10 min (at 60 °C) with a mixture of (0.25% w/v) Coomassie blue-Red 250 (CB-R250) in a methanolic solution (MeOH: CH₃COOH: DW 5:1:4 v/v) just prepared, for detecting proteins at the infection sites. Then, they were washed three times with the same methanolic solution (3 min at room temperature). Due to staining of chloroplasts, a blue background could not be avoided, but proteins stained violet-blue (WOLF and FRIC', 1981) to purple-red if basic ones (WILSON, 1992).

Cycloheximide assays

Fifteen stem segments bordering the youngest leaves, and fifteen fully-expanded first leaves from cashew clone CCP-1001 (60-day-old) were excised, and the cut ends of the excised tissue sealed with warm, molten paraffin wax. Two groups of five stem segments and five leaves (abaxial surface) were placed inside plastic boxes (20 cm × 12 cm × 4 cm), on filter paper moistened either with an aqueous solution of 0.6 mM cycloheximide or with SDW. The samples were immediately inoculated with isolate 905 of *C. gloeosporioides* and incubated as described by López (1999) and López and Lucas (2010a). The third group of samples were treated with cycloheximide but not inoculated (control), to observe later the possible occurrence of phytotoxicity. After 72 h, the two groups of inoculated stem segments and leaves were collected for microscopic examination.

For assessment of penetration sites beneath appressoria, three sections of each sample were clarified, stained with Ab and mounted as described in the staining item. For each replicate, *ca.* 100 sites (appressoria formed) were examined by LM, being classified as follows: 1) no attempted penetration and cell wall apposition; 2) wall apposition formation with successful penetration; 3) wall apposition formation with successful penetration; 4) penetration without cell wall apposition. Penetration

was considered successful if encased hyphae overcame papillae or the infection peg (IP) immediately enlarged into the cell lumen. Total sites in each category for the three replicates of the five samples were determined. The leaf samples were further incubated up to 168h after inoculation, and then examined to assess disease severity, as described by López and Lucas (2010). This experiment was repeated three times.

Results and discussion

Infection and colonization process on young leaves and stems

There was no observed difference between susceptible (isolate LARS-910 × CCP-76, CAP-14 or CCP-1001; isolate 905 × CCP-76 or CAP-14) and resistant (LARS-905 × CCP-1001) interactions of *C. gloeosporioides* complex-cashew leaf/stem tissues during the pre-penetration phase. After penetration, the main differences were the timing, number of invaded cells and extent of the host cell response to infection (Tables 1 and 2).

Table 1. Time course of development (%) of the isolates LARS-905 and 910 of the complex *C. gloeosporioides* on glass slides^a and leaves (abaxial face) of the clones CCP-76, CCP-1001 and CAP-14 of *A. occidentale*.

Hours after Inoculation	Development Phase	910 × CCP76	910 × CCP1001	910 × CAP14	905 × CCP76	905 × CCP1001	905 × CAP14	Gl. Slide ^a 910	Gl. Slide ^a 905
12	Germination	93	92	90	91	89	94	90	91
	Appressorium formed ^b	75	77	72	73	72	76	72	71
18	Germination	95	94	96	97	90	95	95	93
	Appressorium formed ^b	86	85	83	82	80	83	87	85
	Papillae ^c	21	20	23	23	27	24	-	-
24	Appressorium formed ^b	90	89	90	89	88	93	91	89
	Papillae ^c	22	23	26	25	30	27	-	-
38	Papillae ^c	36	38	40	39	42	40	-	-
48	Papillae ^c	47	47	48	49	52	52	-	-
	Large Primary Hypha (LPH) ^d	16	17	18	17	13	19	-	-
66	Papillae ^c	69	71	71	73	75	73	-	-
	LPH ^d	33 ^f	32 ^f	34 ^f	32 ^f	17 ^g	35 ^f	-	-
74	LPH ^d	37 ^f	38 ^f	37 ^f	35 ^f	23 ^g	31 ^f	-	-
	Invasion of other cells Secondary hypha (SH) ^e	2-3 cells 15	2-3 cells 14	2-3 cells 14	2-3 cells 13	1-2 cells ^g g	2-3 cells 15	-	-
96	Invasion of other cells SH ^e	3-5 cells 18 ^h	3-5 cells 16 ^h	3-5 cells 17 ^h	3-5 cells 17 ^h	1-2 cells ^g g	3-5 cells 19 ^h	-	-
114	lesion development	+	+	+	+	-	+	-	-
	Growth in necrotic tissues	+	conidiat. (acervuli and setae)	conidiat. (acervuli and setae)	+	-	+	-	-

^a A minimum of 100 conidia, appressoria or reaction sites were recorded in each of 3 replicates of 5 samples per interaction per time. ^b Percentage from the germinated conidia. ^c Percentage from the appressoria formed and/or attempts of penetration. Papillae (mainly dome-like shape) were observed during cuticular penetration and thin primary hypha (TPH) formation, in all the interactions. ^d Percentage from attempts of penetration (with or without papillae). LPH formed in the absence of papilla were more common in compatible interactions. ^e Percentage from the LPH formed; ^f When multiple penetration attempts occurred in the same cell, its rapid collapse restricted the TPH advance; ^g Penetration restricted to the first cell containing LPH; ^h Because of the rapid and intense browning of the invaded cells, SH were not recorded in whole tissues, but in semi-thin resin embedded sections (LM). Gl. Slide = glass slide; conidiat. = conidiation.

Table 2. Time course of development (%) of the isolates LARS-905 and 910 of the complex *C. gloeosporioides* on glass slides^a and stem segments of the clones CCP-76, CCP-1001 and CAP-14 of *A. occidentale*.

Hours after Inoculation	Development Phase	910 × CCP76	910 × CCP1001	910 × CAP14	905 × CCP76	905 × CCP1001	905 × CAP14	Gl. Slide ^a 910	Gl. Slide ^a 905
12	Germination	91	89	93	89	85	92	90	91
	Appressorium formed ^b	75	73	68	70	69	72	72	71
18	Germination	94	92	96	93	91	96	95	93
	Appressorium formed ^b	81	83	80	80	79	82	87	85
	Papillae ^c	23	25	21	25	29	23	-	-
24	Appressorium formed ^b	88	86	92	90	89	94	91	89
	Papillae ^c	29	33	30	31	36	32	-	-
38	Papillae ^c	39	41	42	41	47	44	-	-
48	Papillae ^c	50	52	53	52	57	53	-	-
	Large Primary Hypha (LPH) ^d	20	22	25	21	17	23	-	-
66	Papillae ^c	71	72	73	73	78	75	-	-
	LPH ^d	37 ^f	35 ^f	36 ^f	37 ^f	21 ^f	35 ^f	-	-
74	LPH ^d	43 ^f	40 ^f	42 ^f	41 ^f	25 ^g	41 ^f	-	-
	Invasion of other cells	2-3 cells	2-3 cells	2-3 cells	2-3 cells	1-3 cells ^g	2-3 cells	-	-
	Secondary hypha (SH) ^e	20	19	23	18	^g	20	-	-
96	Invasion of other cells	3-5 cells	3-5 cells	3-5 cells	3-5 cells	1-3 cells ^g	3-5 cells	-	-
	SH ^e	23 ^h	22 ^h	25 ^h	22 ^h	^g	21 ^h	-	-
114	lesion development	+	+	+	+	-	+	-	-
	Growth in necrotic tissues	+	conidiat. (acervuli and setae)	conidiat. (acervuli and setae)	+	-	conidiat. (acervuli and setae)	-	-

^a A minimum of 100 conidia, appressoria or reaction sites were recorded in each of 3 replicates of 5 samples per interaction per time. ^b Percentage from the germinated conidia. ^c Percentage from the appressoria formed and/or attempts of penetration. Papillae (mainly dome-like shape) were observed during the cuticular penetration and thin primary hypha (TPH) formation, in all the interactions. ^d Percentage from attempts of penetration (with or without papillae). LPH formed in the absence of papilla was more common in compatible interactions. ^e Percentage from the LPH formed; ^f When multiple penetration attempts occurred in the same cell, its rapid collapse restricted the TPH advance; ^g Penetration restricted to the first cell containing LPH; ^h Because of the rapid and intense browning of the invaded cells, SH were not recorded in whole tissues, but in semi-thin resin embedded sections (LM). Gl. Slide = glass slide; conidiat. = conidiation.

In pre-penetration stage, it was possible to see that conidia were longer than the length of stomatal pores of the small cashew leaf epidermal cells (**Figure 1a**). An extracellular matrix helped the appressoria to adhere firmly to the host surface. Direct penetration through the trichomes and cuticle of epidermal cells (**Figure 1b**) was the most common infection route, but the fungus also penetrated through stomatal pores (**Figure 2**). Intramural invasion was rarely found, except after mechanical injury, when it was common. Mechanical injury invariably resulted in the activation of “quiescent” infections in all interactions and even in mature tissues to produce necrotic lesions.

Although infection pegs (IP) could not be very well visualized with the leaf clearing/stain technique (LM), they were evident in semi or ultra-thin sections. Penetration of trichome cells was always associated with a hypersensitive response, being also described in the interactions for northern jointvetch–*C. gloeosporioides* f.sp. *aeschynomene*, tea–*G. theasinensis* and alfalfa–*C.*

trifolii (TEBEEEST et al., 1978; ANDO and HAMAYA, 1986; PORTO et al., 1988). Zainudin and Omar (1988) and Zakaria et al. (2000) also observed stomatal penetration in rubber by isolates of the *C. gloeosporioides* complex, as Kumar et al. (2001) and Panday et al. (2012) have seen in mulberry and onion leaves, respectively.

In both interactions of the cashew–*C. gloeosporioides* complex system, epidermal cells beneath melanized appressoria initially responded to the IP (24–48 h) by changing the existing wall chemistry with a continuous deposition of yellow-orange (LM) electron-dense (TEM) wall material, forming halos and papillae of different shapes and sizes (**Figure 3**). Where the IP extended (48–74 h) through the epidermal cell wall (ECW) and papilla, it formed a single (**Figure 4**) or sometimes forked (**Figure 7a**) thin primary hypha (TPH). In compatible interactions, the first cell containing an encased TPH always remained alive in the earlier stages, as indicated by its ability to plasmolyse normally (**Figure 3**).

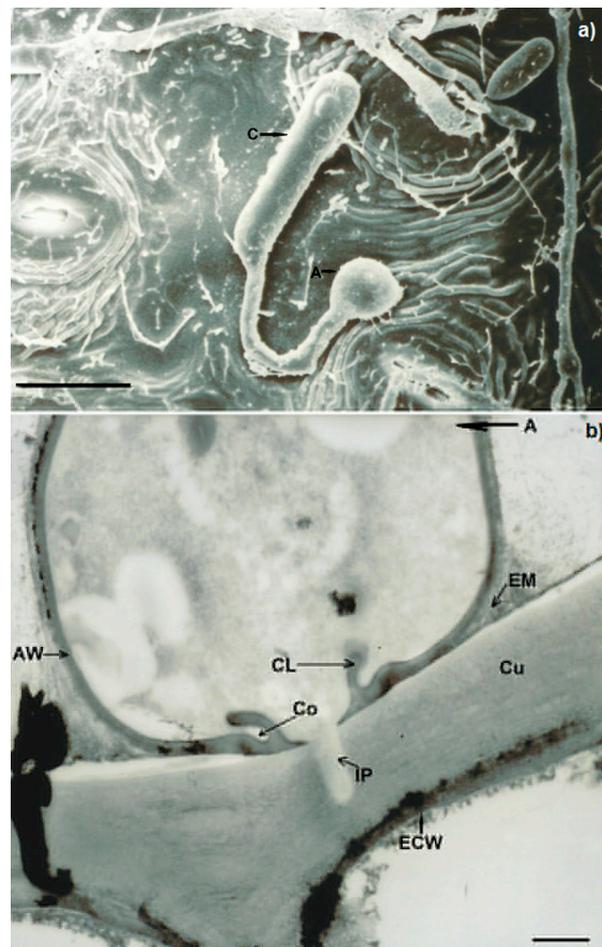


Figure 1. Compatible interaction between the isolate LARS-910 of the *C. gloeosporioides* complex and the abaxial surface of cashew leaf clone CCP-76. **A)** SEM of conidia (C) and non-sessile appressorium (A), 30 h after inoculation ($\times 2,720$, Bar = 10 μm); **b)** TEM of ultra-thin near-median section of appressorium (A) penetrating through the cuticle (Cu) of the epidermal cell wall (ECW), 48 h after inoculation, showing the cone (Co) formed with wall material of the newly formed infection peg (IP) on the collar (CL), the extra-cellular matrix (EM) covering the appressorial wall (AW), the non-deformation of the Cu and the deposition electron-dense of material on the ECW (stain with uranyl acetate/lead citrate, $\times 15,000$, Bar = 1.0 μm).

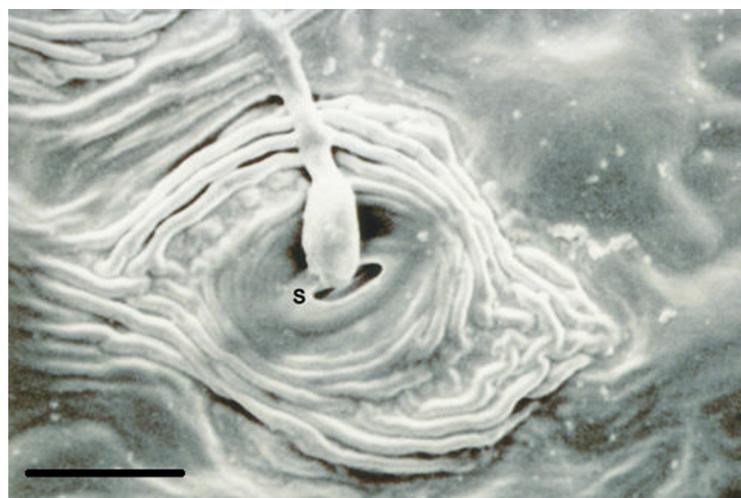


Figure 2. SEM of the abaxial surface of cashew leaf (CCP-1001), 30 h after inoculation with the isolate LARS-905 of *C. gloeosporioides* complex (incompatible interaction), in which a hypha forming a subglobose appressorium penetrated stomatum ($\times 2,720$, Bar = 10.0 μm).

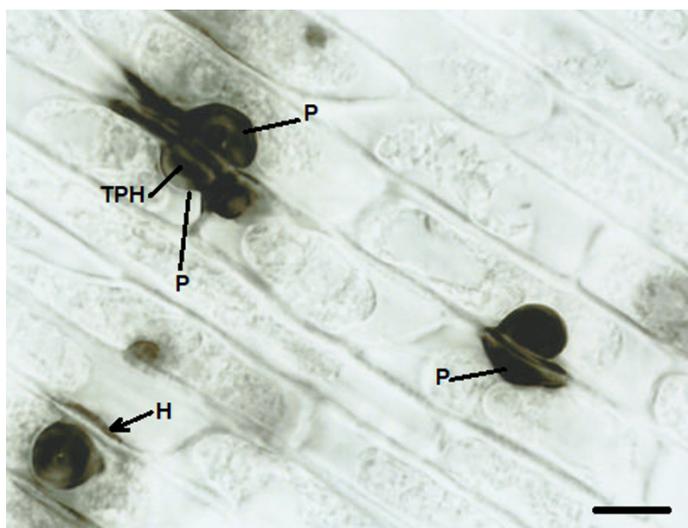


Figure 3. LM of the stem epidermal cells of cashew clone CCP-76, 48 h after inoculation with the isolate LARS-910 of the *C. gloeosporioides* complex (compatible interaction), and then treatment with a plasmolysing solution of KNO_3 , showing papilla (P) and plasmolysed cells (Bar = 12.5 μm). Note halos (H), different wall appositions such as papillae (P), and intracellular hypha (arrow) from the infection site, impregnated with orange material.

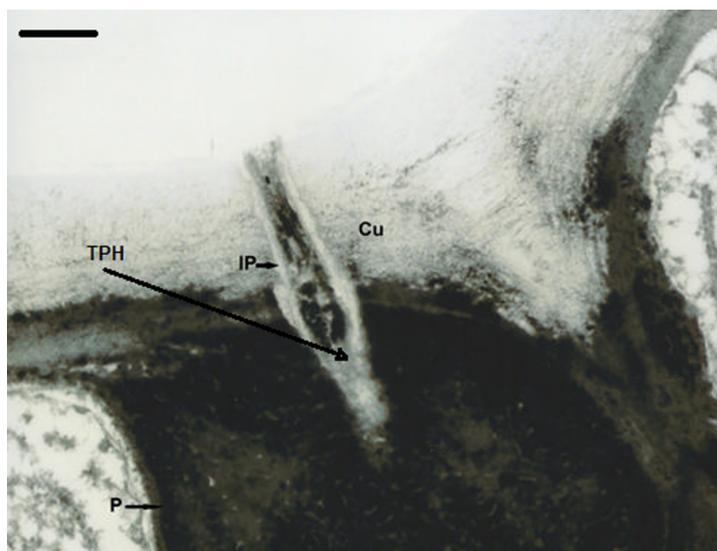


Figure 4. TEM of ultra-thin section of epidermal cell of abaxial surface of cashew leaf (CCP-1001), 48 h after inoculation with the isolate LARS-910 of *C. gloeosporioides* complex (compatible interaction), stained with uranyl acetate/lead citrate. Note the non deformation of the cuticle (Cu), the removal of the appressorium during the resin embedding procedure, and a thin primary hypha (TPH) expanding from an infection peg (IP) during the penetration of the papilla ($\times 20,000$, Bar=2.0 μm).

In both interactions, when a papilla with a hypersensitive response (HR) was not formed quickly enough, it was possible to see the post-penetration events. However, in the resistant combination, only a small proportion of infected cells containing TPH did not immediately restrict them, since the majority are able to plasmolyse too (**Figure 5a**). Usually, a HR was quickly induced in the first invaded and adjacent cells, restricting the development of the fungus (**Figure 5b**). On the other hand, in compatible combinations, the TPH immediately enlarged from the IP, forming the large primary hypha (LPH) that totally filled the lumen of the invaded cell (**Figure 6a**), which sometimes lost organization and

stopped to plasmolyse, just before a thinner secondary hypha (TSH) had been formed and grew through thickened and electron-dense cell walls of adjacent cells (**Figure 6b**). In such necrotic cells, the granulation containing the yellow-orange pigment apparently burst and spread, simultaneously to the degeneration of organelles and membrane.

In the resistant interaction, however, the formed papillae could extend through the adjacent cells more quickly than the encased-TPH developed (**Figure 7a**), and they very rarely enlarged to a LPH, being possible to detect HR as soon as it is eventually formed (**Figure 7b**).

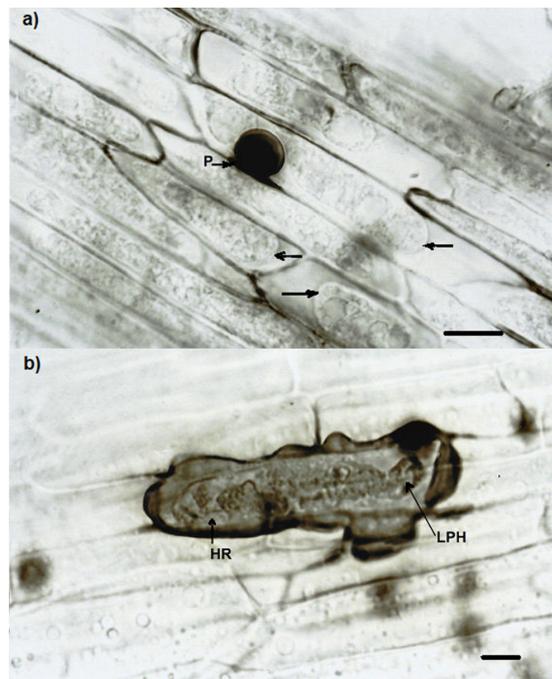


Figure 5. LM of the incompatible interaction of the isolate LARS-905 of the *C. gloeosporioides* complex and stem epidermal cells of cashew clone CCP-1001. **a)** 66 h after inoculation and then treatment with a plasmolysing solution of KNO_3 , showing papilla (P) and plasmolysed cells (Bar = 12.5 μm). **b)** LM 74 h after inoculation, with a rapid hypersensitive reaction (HR) blocking the further development of the fungus into adjacent cells (Bar = 8.0 μm).

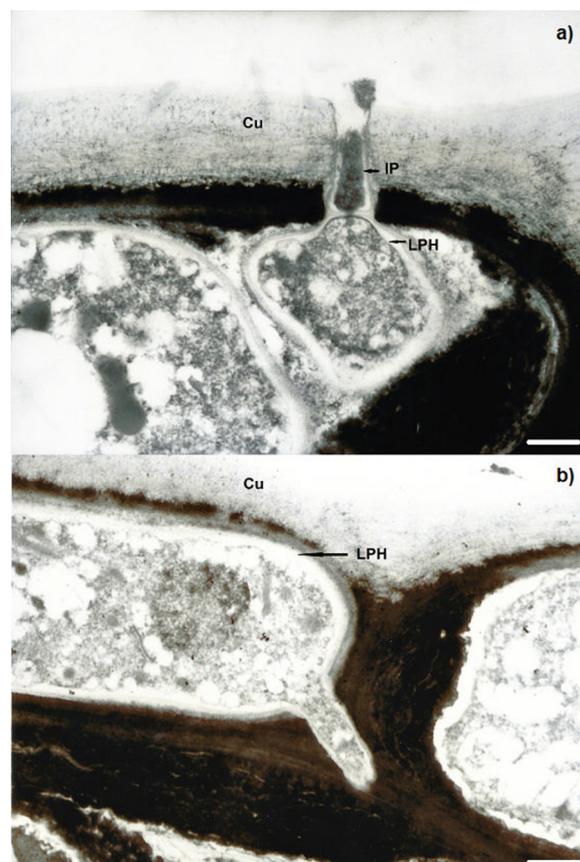


Figure 6. TEM of ultra-thin section of compatible interactions of the abaxial surface of cashew leaf (CCP-76) with *C. gloeosporioides* complex (stained with uranyl acetate/lead citrate). **a)** 74 h after inoculation with isolate 910, showing an infection site where a papilla was not formed and the infection peg (IP) enlarged as a large primary hypha (LPH) just after penetration through the cuticle (Cu) and epidermal cell wall (ECW) ($\times 8,000$, Bar=4.5 μm). **b)** 74 h after inoculation with isolate 905, showing a LPH that filled the lumen of the epidermal cell and started to penetrate through the thicker cell wall, to invade an adjacent healthy cell. Note the constriction of the LPH while passing through the electron-dense material on the cell walls around the infection site ($\times 8,000$, Bar=4.5 μm).

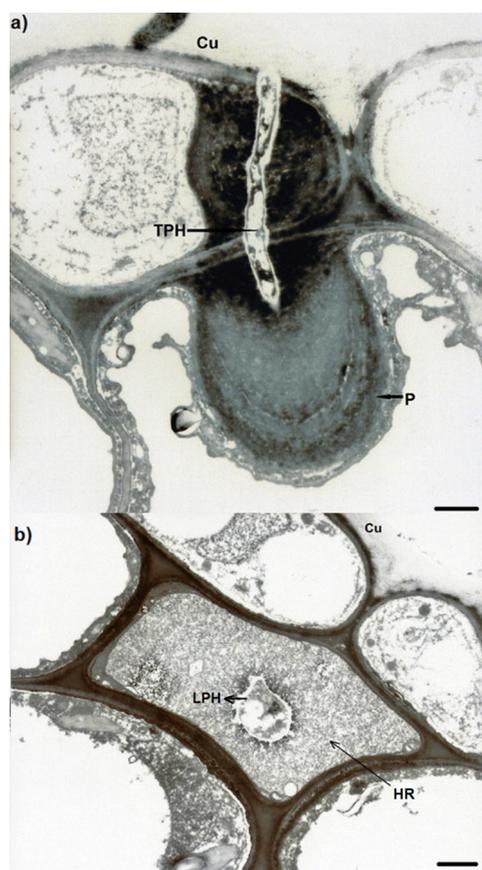


Figure 7. TEM of ultra-thin section of epidermal cell of abaxial surface of cashew leaf (CCP-1001) inoculated with the isolate LARS-905 of *C. gloeosporioides* complex (incompatible interaction), stained with uranyl acetate/lead citrate. **a)** Note the removal of the appressorium during the resin embedding procedure; 66 h after the inoculation, an encased thin primary hypha (TPH) penetrated through the epidermal cell wall (ECW) and through the formed papilla (P), which extended to another adjacent cell, and the invaded cells could still plasmolyse ($\times 7,000$, Bar = 4.0 μm). **b)** 74 h after inoculation, a rapid hypersensitive response (HR) was observed restricting the large primary hypha (LPH) emerging from a previously invaded cell. Note the electron-dense granulation inside the cell and the more electron dense walls of the hypersensitive and adjacent cells ($\times 5,000$, Bar = 3.0 μm).

TSH was not observed in this interaction. In the later stages of the compatible infections (114-182 h), acervuli and conidiation could be observed, and superficial hyphae, or hyphae that emerged from

collapsed cells, grew across the surface and directly penetrated subepidermal parenchyma tissue (**Figures 8-9**).

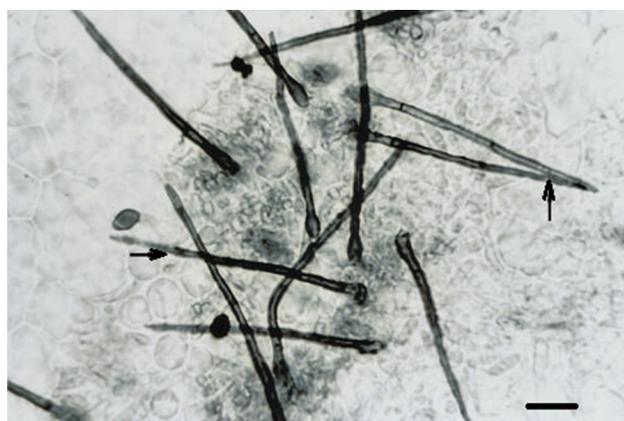


Figure 8. LM of the abaxial surface of cashew leaf (CCP-76) 120 h after inoculation with isolate LARS-905 of the *C. gloeosporioides* complex, showing the presence of sterile setae (compatible interaction, stained with Aniline blue, Bar = 15.6 μm).

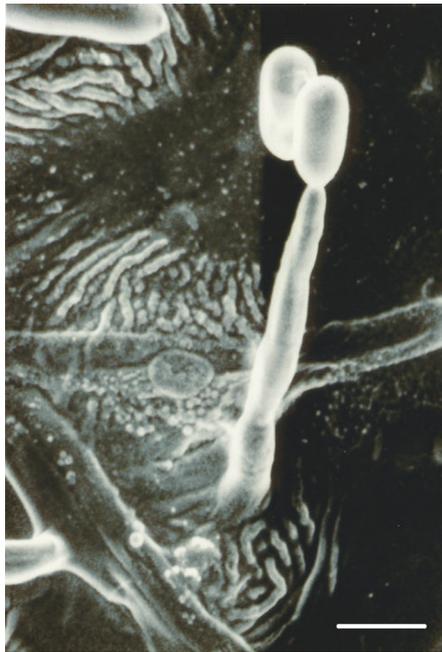


Figure 9. SEM of the abaxial surface of cashew leaf (CCP-1001) 96 h after inoculation with isolate LARS-910 of *C. gloeosporioides* complex, showing fertile seta emerging through subsidiary epidermal cell (Bar = 5 μ m) in the later stages of a compatible interaction.

Pandey *et al.* (2012a), studying onion leaves infected with isolate of the *C. gloeosporioides* complex, also showed that 6 h after the inoculation, a germ tube emerged from the conidium of this fungus and a globular shaped appressorium formed at the tip, maturing for 24-48 h; then, an infection hypha emerged through a pore at its base and penetrated directly the host cuticle, forming a primary hypha, to whom the onion leaf cell also responds by producing the papilla. In such leaves, the authors also could see, as in the present work, simultaneous penetration of the host through stomata. According to the authors, from 48-72 h, primary hyphae in onion cells started to branch out to form secondary hyphae within the epidermal cells followed by a massive growth of both intra- and intercellular hyphae leading to the development of small whitish and water-soaked lesions, and at 72-96 h after inoculation, intra- and intercellular hyphae radiated from cell to cell, resulting in the formation of acervuli. After that, typical onion anthracnose symptoms, with salmon coloured mucilaginous spore matrix were observed on the infected leaf surface, as was also seen in the infected leaves of susceptible cashew studied here. In addition to these studies, when the same group (PANDEY *et al.* 2012a) worked with leaves of *Mangifera indica* susceptible to one isolate of the *C. gloeosporioides* complex, it was found that hyphal invasion occurred intra and intercellularly until 72 h after the inoculation, with rapid collapse of the mesophyll cells and swelling of epidermal cell walls, and almost all the initially invaded cells became necrotic 96 h after inoculation, which corroborates with what was observed in this study.

Therefore, the formation of wall appositions and hypersensitive cell death are responses that could contribute to the epidermal resistance of cashew against the invasion of the studied isolates of the *C. gloeosporioides* complex, besides the individual clonal phenology. An important fact to note is that the isolates used in this study were previously identified through their morphophysiological and genetical (rDNA large subunit) aspects as a member of *C. gloeosporioides* complex, as well as 34 other isolates with aggressiveness against cashew trees (López and Lucas, 2010), even if obtained from lesions in other associated hosts (cross-pathogenicity). However, the diversity of survival strategies and life cycles of *Colletotrichum* species are characteristics responsible for their high taxonomic complexity, and for this reason the reclassification of species of this genus was established (CANNON *et al.* (2012). By 2018, 218 species had already been recognized in the same Genera, and the majority were subclassified in 12 species complexes and 2 others were considered individual species (JAYAWARDENA *et al.*, 2016; GUARNACCIA *et al.*, 2017; VELOSO *et al.* 2018), so that, currently, *C. gloeosporioides* is considered a species complex for different hosts. In the case of cashew trees, Comé (2014) and then Veloso *et al.* (2018) evidenced, by multilocus genetic analysis, six and then seven species, respectively, of this complex, capable of infecting this host, and it is also probable that both isolates used in the present study, although belonging to the same complex, may currently correspond to phylogenetically different species, requiring confirmation by the multifocus analysis. On the other hand, for the purpose of this work,

in which an interaction incompatible with an isolate originally from another host plant and three compatible ones, with an isolate obtained originally from cashew anthracnose lesions, this information does not affect the histological results reported here.

Histochemical studies on papillae in cashew leaves and stems

Table 3 shows the colour reaction of infection sites in stems and leaves of infected cashew seedlings stained as described in the methodology.

Under bright field illumination, wall appositions and the lumen of hypersensitive infected cells were yellow-orange (**Figure 3**), and after aniline blue (Ab) treatment and examination under UV-light, the inner

surface of the cell walls beneath appressoria and papillae, in both interactions, gave positive results for callose. However, callose itself was not the only component of the papillae, since granular deposits in the recently-formed outer layer did not fluoresce with Ab and stained purple (bright field) with Tb-O treatments, a result negative for lignin-like compounds, but positive for pectin, cellulose, hemicellulose (O'BRIEN et al., 1964; HERR, 1992), proteinaceous material (CLOUD and DEVERALL, 1987) or opaline silica (HEATH, 1979). The other histochemical tests for lignin evidenced that phenolic material of different types was detected (**Table 3**). Autofluorescence, a characteristic of lignin-like compounds, was not observed at any of the penetration sites or in the lumen of hypersensitive cells.

Table 3. Histochemical colour reactions of papillae, halos and hypersensitive cells of cashew leaves or stems inoculated with isolates LARS-905 and 910 of the *C. gloeosporioides* complex.

Test	Expected Colour	Reaction
Aniline blue (AB)	Callose = green-yellow fluorescence	+
AB- Toluidine blue (TB)	Callose = green-yellow fluorescence	+
Autofluorescence	Lignin = fluorescence	-
TB	Lignin = blue-green Callose = unstained Hemicellulose, cellulose, pectin = purple-red Silicates and polysulfates = purple-red Proteinaceous material = purple-red	Purple-red
TB - CaCl ₂	Lignin = blue; other compounds = unstained	Unstained*
Phloroglucinol – HCl	Guayacyl lignin = red Syringaldehyde lignin = unstained	Unstained*
Phloroglucinol:CaCl ₂ -HCl	Lignin = deep orange to red	Unstained*
Chlorine water: Na ₂ SO ₃	Guayacyl lignin = brown Syringyl lignin = red Catechol, resorcinol, quinol = unstained Tannic acid, methyl gallate, pyrogallol = pink	Unstained*
KMnO ₄ – HCl - NH ₄ OH	Syringyl-propane lignin = purple to red-brown	Unstained*
Diazotized <i>p</i> -nitroaniline	Phenols with an <i>ortho</i> or <i>para</i> free position = yellow-orange to brown Ferulic and sinapic acids = pink Lignin = orange-red to red	Unstained*
<i>p</i> -nitroaniline-tetrafluoroborate	Phenols with an <i>ortho</i> or <i>para</i> free position = orange-red. Red-violet after NaOH	Unstained*
FeCl ₃	Pectin-tannin, hydroxamic acid derivatives = reddish-brown; Catechol = blue-green	reddish-brown (healthy cells = blue)
Nitroso Reaction	Anthocyanins, anthoxanthins = yellow Gallotannins = yellow, orange-red Flavonols, ellagitannins, catechins and catechol derivatives (hydroxy-naftoquinones) = cherry to red-brown	Deep Purple
Vanillin: HCl	Condensed tannins, dihydrochalcones, anthocyanins, resorcinol = pink to brick-red Catechol, pyrogallol, hydroquinone, phloroglucinol, hydrolysable tannins = unstained	Brick red (fading to orange). Red in blue light excitation (filter barrier 520 nm)
Syringaldazine- H ₂ O ₂	Laccase = purple before adding hydrogen peroxide Peroxidase = purple after adding hydrogen peroxide	Purple with H ₂ O ₂
Coomassie Blue (Cb)	Acid and neutral proteins = blue violet Histones, PRPs, HPRPs = purple-red	Purple-red
NaOH (0.5-10 N)	Dissolves protoplasm; saponifies suberin Cyanidin derivatives = blue	Unstained* (healthy cells = blue)
H ₂ O ₂ : CH ₃ COOH	Dissolves brown quinones	Pale-yellow

* Appositions and hypersensitive cells = yellow-orange before stain, and orange after stain.

Different dyes increased the colour intensity of the infection sites to dark orange (bright field), and according to the phloroglucinol: HCl, phloroglucinol: $\text{CaCl}_2 \rightarrow \text{HCl}$ and chlorine-sodium sulphite tests, this could correspond to the presence of syringyl-lignin with syringyl-propane groups. In such cases, papillae and hypersensitive cells (48-120 h after inoculation) treated with $\text{KMnO}_4\text{-HCl-NH}_4\text{OH}$ should stain purple-red, but this was not seen. The diazotised-*p*-nitroaniline and *p*-nitrobenzene tetrafluoroborate- tests were positive for phenols (mono or polyhydroxy) with a *para* or *ortho* position free, but not necessarily lignin. At infection sites, the ferric chloride, vanillin and nitroso reactions were positive for flavonols, pectin bound to tannins or condensed tannins.

Whereas most reports in the literature assume that proteins stain blue-violet with Cb-R250, the wall appositions and hypersensitive cells of cashew leaves stained red-purple. Rosenthal et al. (1988) and Wilson (1992), however, found that histones, rubrophilin (a brain protein), collagen and other HRGPs and PRPs, which have a high affinity for tannins, dye red with Cb-R250. Woof *et al.* (1968) reported that proteins linked to di- or polyhydroxyphenols, independent of the pH of the interaction, produce different spectra with the individual compounds. Hagerman and Butler (1989) asserted that efforts must be made to remove all tannin from plant extracts before colorimetric tests with Cb, since the dye reacts to tannin as well as to protein. After mechanical or biotic wounding, as caused by insects or the fungus *Botryodiplodia theobromae* (syn. *Lasiodiplodia theobromae*), resin ducts of cashew trees, that are also present in their leaves (METCALFE and CHALK, 1950), secrete a gum formed by arabinogalactan proteins (AGPs), phenols and enzymes (polyphenoloxidase, peroxidase, chitinase and proteinase inhibitors) (MARQUES and XAVIER-FILHO, 1991; PAULA and RODRIGUES, 1995), and although an aqueous solution of this gum (33%) did not affect the mycelial growth *in vitro* of *C. gloeosporioides*, it inhibited several microorganisms (MARQUES et al., 1992).

Phenol oxidation of syringaldazine in the presence of H_2O_2 produces the intensely purple tetramethoxyazo-*p*-methylenequinone. This histochemical test for peroxidase was positive in all the reaction sites and hypersensitive cells, 48-120 h after inoculation, with all the interactions. Walls of unchallenged cells or non-inoculated controls did not exhibit peroxidase activity. Although some authors have associated oxidation of syringaldazine with lignin formation (LEWIS and YAMAMOTO, 1990), the oxidase activity detected is not necessarily specific to lignin residues, since laccase from extracellular mucilage of *C. graminicola* complex conidia has also been detected with this substrate (ANDERSON and NICHOLSON, 1996). This glycoprotein mucilage, which

has high affinity for binding condensed tannins, protects spores from inhibition of germination by polyphenols (NICHOLSON et al., 1986). While laccase can oxidise dihydroxyflavonols (ANDERSON and NICHOLSON, 1996), plant peroxidases are implicated in the cross-linking of cell wall components (polyphenols, HRGP and pectin), synthesis of *o*-quinones (HARRISON et al., 1995) and conversion of dihydroxyphenols to free radicals. The later may undergo reactions with cellular constituents and have toxic activity against microorganisms (BELL, 1981).

Oxidation and condensation of *o*-dihydroxyphenolic compounds (such as catechin, gallocatechin and their proanthocyanidin derivatives) is generally associated with resistance and characterised by the formation of brown *o*-quinones throughout the cell walls and collapsed protoplasts (BELL, 1981). In this study, necrotic tissues in susceptible interactions exude an intense brown pigment from the collapsed cells. When drops of NaOH (0.5-10 N) were placed on the sections, rather than dissolving the yellow-orange pigmentation at infection sites, the healthy tissue became blue (dihydroxyphenol derivatives). Oxidative bleaching (H_2O_2 : CH_3COOH) for 30 min also only caused a partial loss of the pigment intensity to pale yellow and none of the sections showed fluorescence. Increases in the time of treatment up to 90 min caused an overall tissue degeneration. WHEELER et al. (1978) found that treatment with H_2O_2 : CH_3COOH , for up to 4 h, did not completely remove dihydroxyphenol and catechol melanins from the matrix and outer cell wall of microsclerotia of *Verticillium dahliae*.

When the complex tannin-protein-quinone is formed, plant cell walls remain resistant to hydrolysis for extended periods of time (HARBORNE, 1980). Plant *o*-quinones (melanins) by themselves, showed extremely weak electron density in ultrastructural studies with French beans (HEATH, 1979). However, as the condensed tannins, which bind together starch or pectic compounds and form hydrogen bonds with structural proteins (MULLINS and LEE, 1991), quinone residues form covalent bonds with free amino groups of HRGPs. Tannins and *o*-quinones can be deposited in the cell walls, serving as scavengers for electron dense silicates, inactivating microbial extracellular enzymes (BELL, 1981).

As described previously, examination of leaf-samples after conventional preparation for electron microscopy (involving heavy metal staining) revealed typical electron-dense deposits on the inner surface of the cells walls adjacent to appressoria. The appressorial walls, which contain polymeric melanins, appeared less electron dense than the ECWs of the infected leaves (**Figure 1b**). This may indicate that not only accumulation of the common organic constituents (proteins, carbohydrates

and phenols) restricted the passage of electrons through the specimen after staining, but probably also deposits of some elements of high atomic weight. Silicon is a common element in most soils where cashew is grown, and it can be easily assimilated and stored as amorphous opaline silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$). It is energetically cheap for defence, especially against herbivores, and high amounts

of it have been detected in ash of cashew gum (9.3 mg/g ash) and of other Anacardiaceae gum (PINTO et al., 1995). The base of the soft trichomes of young leaves of some Anacardiaceae accumulates silicon (LUCAS; TEAFORD, 1995), besides tannins and alkyl-salicylic acid derivatives (anacardic acids).

Table 4 show the effect of cycloheximide on the formation of cell wall appositions and tissue colonization.

Table 4. Effect of cycloheximide on wall apposition formation and direct penetration in epidermal cells of stem segments of *A. occidentale* (CCP-1001) inoculated with the isolate LARS-905 of the *C. gloeosporioides* complex.^a

Treatment	Penetration attempts (appressoria formed)	Papillae at penetration attempts	Penetration without papillae	Penetration with Papillae	Neither papillae or penetration
SDW	1432	1102	141	98	92
Cycloheximide	1398	58	1129	84	127

^a Samples were floated on sterilized deionized water (SDW) or cycloheximide solution (0.6 mM), and infection sites examined 72 h after inoculation. A minimum of 100 appressoria in each of three strips of five stems were recorded.

Detached leaves or stem segments from cashew clone CCP-1001 became susceptible to the pathogen when floated on cycloheximide (0.6 mM) solution immediately prior to inoculation, and the tissue collapsed and exuded brown pigment in advanced stages of infection. When stem segments were floated on SDW for 72 h, *ca.* 77% of the epidermal cells beneath appressoria showed papillae, *ca.* 10% showed penetrations without papillae, *ca.* 7% showed penetrations through the appositions and *ca.* 6% showed no wall apposition and no penetration (**Table 4**). From the reaction sites of cycloheximide treated tissue, only 4 % of the penetration attempts showed appositions, while *ca.* 81% showed penetration without apposition, *ca.* 6% showed penetration through apposition and *ca.* 9% showed no wall apposition and no penetration. Therefore, cycloheximide may have inhibited the synthesis of proteins or enzymes involved in the formation of wall appositions in cashew tissues, and such appositions appear to be important for the active process of epidermal resistance.

Taken together, results of the different histochemical tests suggested that polysaccharides (callose, pectin, hemicellulose and cellulose), proteins (such as hydroxyproline rich glycoproteins, HRGPs), peroxidase and phenolics/tannins are produced and added to stored silicon in response to attempts of penetration in all the observed interactions. The formation of specific inhibitory metabolites at the site of wall apposition, might account for the unsuccessful penetration attempts, and probably varies in timing and intensity, permitting some attempts to succeed.

Conclusions

The cyto-histochemical studies described here, regarding the compatible interactions (LARS-910 × CCP-76, CCP-1001 and CAP-14; LARS-905 × CCP-76 and CAP-14) or resistant combination (LARS-905 × clone 1001) involving isolates of the *C. gloeosporioides* complex and leaves and stems of cashew seedlings, revealed that prior to penetration there are no significant differences between them. In compatible combinations, after the formation of melanized appressoria (24-48h after inoculation), penetration generally occurred from the infection peg (IP) through the cuticle and epidermal cell walls and trichomes. In the resistant combination, the same thing happened, although with a lower frequency and with a longer period of time to form the appressoria (48-74h). Less commonly, direct penetration of the fungus germ tubes through open stomata, without production of appressoria and without affecting the host's cuticle, was observed in both combinations. Very rarely, intramural invasion took place, except after mechanical injury, when it was common. Mechanical injury invariably resulted in the activation of "quiescent" infections in all interactions and even in mature tissues to produce necrotic lesions.

After penetration, no special infection structure was observed in both interactions, although in the compatible combination, the thin primary hypha (TPH) from the developed IP, enlarged in the first invaded epidermal cell, forming a broad primary hypha (LPH) 36-66 h after inoculation, even when eventually a papilla was later formed around the penetration point. In these early stages of colonization, the invaded cells were activated

to trigger various defense mechanisms, albeit apparently slowly. Then, a yellow-brown lignopolysaccharide-protein-silicon complex filled the lumen of the invaded cells, and thin secondary hyphae (TSH) developed from the LPH, starting penetration of the healthy adjacent cells (66-96h) just before the colonized cell underwent necrosis (hemibiotrophy). In the most advanced stages of infection (114-182 h), the majority of colonized epidermal cells collapsed, and acervuli, setae and conidia were formed.

Histochemical tests suggested that, in both compatible or incompatible interactions, papillae are formed by polysaccharides (callose, pectin, hemicellulose and cellulose), PRPs or HPRGs, phenolics and silicates, with peroxidase activity detected in response to penetration in all of them. In the incompatible interaction (LARS-905 × CCP-1001), the treatment of the studied tissues with cycloheximide inhibited the synthesis of wall appositions and related resistance mechanisms. Therefore, the rapid and intense production of papillae and pigmented lignopolysaccharides, that may also signal adjacent cells to react to the fungus before it reaches them, are important defense mechanisms in the resistant interaction.

Such studies, associated with the phenology of the studied cashew clones in the edafoclimatic conditions of the regions where they are grown in the Brazilian Northeast, should enable the best integrated management of the damaging anthracnose disease.

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References

- ALBUQUERQUE, A.C.; SILVA, A. da. **Agricultura tropical: quatro décadas de inovações tecnológicas, institucionais e políticas**. Brasília (DF): EMBRAPA, 2008. 1337 p.
- ANDERSON, D.W.; NICHOLSON, R.L. Characterization of a laccase in the conidial mucilage of *Colletotrichum graminicola*. **Mycology**, College Park, v.88, p.996-1002, 1996.
- ANDO, Y.; HAMAYA, E. Defense reaction of the plant against infection of the tea anthracnose fungus. **Study Tea**, v.69, p.35-43, 1986.
- BAILEY, J.A.; NASH, C.; O'CONNELL, R.J. Infection process and host specificity of a *Colletotrichum* species causing anthracnose disease of cowpea, *Vigna unguiculata*. **Mycology Research**, Manchester, v.94, p.810-814, 1990.
- BARRETO, A.L.H.; VASCONCELOS, I.M.; GRANGEIRO, T.B.; MELO, V.M.M.; MATOS, T.E.; ELOY, Y.R.G.; FERNANDES, C.F.; TORRES, D.C.; FREIRE Fº, F.R.; FREIRE, F.C.O.; OLIVEIRA, J.T.A. Infection process and host defense responses in compatible and incompatible interactions between cowpea (*Vigna unguiculata*) and *Colletotrichum gloeosporioides*. **International Journal of Plant Science**, Chicago, v.168, p.193-203, 2007.
- BELL, A.A. Biochemical mechanisms of disease resistance. **Annual Review of Plant Physiology**, Palo Alto, v.32, p.21-81, 1981.
- BERVINGSON, D.J.; ARNASON, J.T.; PIETRZAK, L.N. Localization and quantification of cell wall phenolics in European corn borer resistant and susceptible maize inbreds. **Canadian Journal of Botany**, Ottawa, v.72, p.1243-1249, 1994.
- CADENA-GOMES, G.; NICHOLSON, R.L. Papilla formation and associated peroxidase activity: A non-specific response to attempted fungal penetration of maize. **Physiological and Molecular Plant Pathology**, London, v.31, p.51-67, 1987.
- CAMPBELL, W.G.; BRYANT, S.A.; SWANN, G. CLXI. The chlorine-sodium sulphite colour reaction of woody tissues. **Biochemical Journal**, London, v.81, p.1285-1288, 1937.
- CANNON, P.F.; DAMM, U.; JOHNSTON, P.R.; WEIR, B.S. *Colletotrichum* – current status and future directions. **Studies in Mycology**, Utrecht, v.73, p.181-213, 2012.
- CLOUD, A.M.E.; DEVERALL, B.J. Induction and expression of systemic resistance to the anthracnose disease in bean. **Plant Pathology**, Wallingford, v.36, p.551-557, 1987.
- COMÉ, M.J. **Colletotrichum species associated with anthracnose of cashew tree**. 2014. Dissertação (Mestrado) - Universidade Federal de Lavras, Lavras. 2014.

- GUARNACCIA, V.; GROENEWALD, J. Z.; POLIZZI, G.; CROUS, P.W. High species diversity in *Colletotrichum* associated with citrus diseases in Europe. **Persoonia**, Leiden, v.39, p.32–50, 2017.
- HAGERMAN, A.E.; BUTLER, L.G. Choosing appropriate methods and standards for assaying tannin. **Journal of Chemical Ecology**, Dordrecht, v.15, p.1795-1810, 1989.
- HARBORNE, J.B. Plant phenolics. In: BELL, E.A.; CHARLWOOD, B.V. (ed.). **Secondary plant products**. Heidelberg: Springer-Verlag Berlin, 1980, p.329-402.
- HARBORNE, J.B. **Phytochemical methods**. 2nd ed. London: Chapman and Hall, 1991. 288p.
- HARKIN, J.M.; OBST, J.R. Lignification in trees: indication of exclusive peroxidase participation. **Science**, London, v.180, p.296-298, 1973.
- HARRIS, P.J.; HARTLEY, R.D.; BARTON, G.E. Evaluation of stabilized diazonium salts for detection of phenolic constituents of plant cell walls. **Journal of the Science of Food and Agriculture**, New York, v.33, p.516-520, 1982.
- HARRISON, S.J.; CURTIS, M.D.; MCINTYRE, C.L.; MACLEAN, D.; MANNERS, J.M. Differential expression of peroxidase isogenes during the early stages of infection of the tropical forage legume *Stylosanthes humilis* by *Colletotrichum gloeosporioides*. **Molecular Plant-Microbe Interactions**, St Paul, v.8, n.3, p.398-406, 1995.
- HEATH, M.C. Partial characterization of the electron-opaque deposits formed in the non-host plant, french bean, after cowpea rust infection. **Physiological Plant Pathology**, Oxford, v.15, p.141-148, 1979.
- HERR, J.M. New uses of calcium chloride solution as a mounting medium. **Biotechnic & Histochemistry**, v.67, n.1, p.9-13, 1992.
- HOOD, M.E.; SHEW, H.D. Applications of KOH-aniline blue fluorescence in the study of plant-fungal interactions. **Phytopathology**, St Paul, v.86, p.704-708, 1996.
- JAYAWARDENA, R.S.; HYDE, K.D.; JEEWON, R.; LIU, X.H.; LIU, M.; YAN, J.Y. Why it is important to correctly name *Colletotrichum* species? **Mycosphere**, Lund, v.7, p.1076–1092, 2016.
- JENSEN, W.A. **Botanical histochemistry: principles and practice**. London: Freeman and Co., 1962. p.203-205.
- KHATOON, A.; MOHAPATRA, A.; SATAPATHY, K.B. Major diseases of cashew (*Anacardium occidentale* L.) caused by fungi and their control in Odisha, India. **International Journal of Bioscience**, Calabar, v.11, p.68-74, 2017.
- KUMAR, V.; GUPTA, V.P.; BABU, A.M.; MISHRA, R.K.; THIAGARAJAN, V.; DATTA, R.K. Surface ultrastructural studies on penetration and infection process of *Colletotrichum gloeosporioides* on mulberry leaf causing black spot disease. **Journal of Phytopathology**, Berlin, v.149, p.629-633, 2001.
- LATUNDE-DADA, O.; O'CONNELL, R.J.; NASCH, C.; PRING, R.J.; LUCAS, J.A.; BAILEY, J. Infection process and identity of hemibiotrophic anthracnose fungus (*Colletotrichum destructivum*) from cowpea (*Vigna unguiculata*). **Mycology Research**, Manchester, v.100, p.1133-1141, 1996.
- LEWIS, N.G.; YAMAMOTO, E. Lignin occurrence, biogenesis and biodegradation. **Annual Review of Plant Physiology and Plant Molecular Biology**, Palo Alto, v.41, p.455-496, 1990.
- LÓPEZ, A.M.Q.; LUCAS, J.A. Reaction of dwarf cashew clones to *Colletotrichum gloeosporioides* isolates in controlled environment. **Scientia Agricola**, Piracicaba, v.67, n.2, p.228-235, 2010a.
- LÓPEZ, A.M.Q.; LUCAS, J.A. *Colletotrichum* isolates related to anthracnose of cashew trees in Brazil: morphological and molecular description using LSU rDNA sequences. **Brazilian Archives of Biology and Technology**, Curitiba, v.53, p.741-752, 2010b.
- LUCAS, P.W.; TEAFORD, M.F. Significance of silica in leaves to long-tailed macaques (*Macaca fascicularis*). **Folia Primatologica**, Basel, v.64, p.30-36, 1995.
- MARQUES, M.R.; ALBUQUERQUE, L.M.B.; XAVIER-FILHO, J. Antimicrobial and insecticidal activities of cashew tree gum exudate. **Annals of Applied Biology**, Oxford, v.121, p.371-377, 1992.
- MARQUES, M.R.; XAVIER-FILHO, J. Enzymatic and inhibitory activities of cashew tree gum exudate. **Phytochemistry**, New York, v.30, p.1431-1433, 1991. METCALFE, C.R.; CHALK, L. Anacardiaceae. In: METCALFE, C.R.; CHALK, L. **Anatomy of dicotyledons**. London: Oxford University Press, London, 1950. v.1, p.452-462.

- MONTEIRO, F.; ROMEIRAS, M. M.; FIGUEIREDO, A.; SEBASTIANA, M.; BALDÉ, A.; CATARINO, L.; BATISTA, D. Tracking cashew economically important diseases in the West African region using metagenomics. **Frontiers of Plant Science**, New Haven, v.6, p.482, 2015.
- MORIN, L.; DERBY, J.-A.L.; KOKKO, E.G. Infection process of *Colletotrichum gloeosporioides* f.sp. *malvae* on Malvaceae weeds. **Mycology Research**, Manchester, v.100, p.165-172, 1996.
- MULLINS, J.T.; LEE, J.H. Interactions of tannins with enzymes: a potential role in the reduced rate of ethanol fermentation from high-tannin biomass. **Biomass and Bioenergy**, Amsterdam, v.1, p.355-361, 1991.
- NICHOLSON, R.L.; BUTLER, L.G.; ASQUITH, T.N. Glycoproteins from *Colletotrichum graminicola* that bind phenols: implications for survival and virulence of phytopathogenic fungi. **Phytopathology**, St Paul, v.76, p.1315-1318, 1986.
- NORSHIE, P.M. *Colletotrichum gloeosporioides* species complex: pathogen causing anthracnose, gummosis and dieback diseases of cashew (*Anacardium Occidentale* L.) in Ghana. **European Journal of Agriculture and Food Sciences**, Bruxelles, v.12, n.6, p.1-10, 2020.
- O'BRIEN, T.P.; FEDER, N.; MCCULLY, M.E. Polychromatic staining of plant cell walls by toluidine blue O. **Protoplasma**, Edinburgh, v.59, p.368-373, 1964.
- O'CONNELL, R.J.; BAILEY, J.A. Differences in the extent of fungal development, host cell necrosis and symptom expression during race-cultivar interactions between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum*. **Plant Pathology**, Wallingford, v.37, p.351-362, 1988.
- O'CONNELL, R.J.; URONU, A.B.; WAKSMAN, G.; NASH, C.; KEON, J.P.R.; BAILEY, J.A. Hemibiotrophic infection of *Pisum sativum* by *Colletotrichum truncatum*. **Plant Pathology**, Wallingford, v.42, p.774-783, 1993.
- O'CONNELL, R.J.; THON M.R.; HACQUARD S.; AMYOTTE S.G.; KLEEMANN J., *et al.* Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. **Nature Genetics**, New York, v.44, p.1060-1065, 2012.
- OPARKA, K.J. Plasmolysis: new insights into an old process. **New Phytology**, London, v. 126, p.571-591, 1994.
- PANDAY S.S.; ALBERTO R.T.; LABEM S.S. Ultrastructural characterization of infection and colonization of *Colletotrichum gloeosporioides* in onion. **Plant Pathology and Quarantine**, Chiang Rai, v.2, p.168-177, 2012a.
- PANDEY, A.; PANDEY, B.K.; MUTHUKUMAR, M.; YADAVA, L.P.; CHAUHAN, U.K. Histopathological Study of infection process of *Colletotrichum gloeosporioides* Penz and Sacc. on *Mangifera indica* L. **Plant Pathology Journal**, Mansoura, v.11, p.18-24, 2012b.
- PAULA, R.C.M.; RODRIGUES, J.F. Composition and rheological properties of cashew tree gum, the exudate polysaccharide from *Anacardium occidentale* L. **Carbohydrates Polymers**, Amsterdam, v.26, p.177-181, 1995.
- PINTO, G.L.; MARTINEZ, M.; MENDOZA, J.A.; OCANDO, E.; RIVAS, C. Comparison of three Anacardiaceae gum exudates. **Biochemical Systematics and Ecology**, Oxford, v. 23, p.151-156, 1995.
- PORTO, M.; GRAU, C.R.; ZOETEN, G.A.; GAARD, G. Histopathology of *Colletotrichum trifolii* on alfafa. **Phytopathology**, St Paul, v.78, p.345-349, 1988.
- REEVE, R.M. Histological and histochemical changes in developing and ripening peaches. I-The catechol tannins. **American Journal of Botany**, Baltimore, v.46, p.210-217, 1959.
- REYNOLDS, E.S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. **Journal of Cell Biology**, New York, v.17, p.208-213, 1963.
- ROSENTHAL, H.L.; BERGHER, R.A.; TYLER, A.N.; MOORE, B.W. Isolation of a component from commercial Coomassie brilliant blue R-250 that stains rubrophilin and other proteins in polyacrylamide gel. **Biochimica et Biophysica Acta**, Amsterdam, v.965, p.106-113, 1988.
- SATAPATHY, R.R.; BEURA, S.K. Management of *Colletotrichum gloeosporioides* (Penz.) causing cashew anthracnose through botanicals. **International Journal of Current Microbiology and Applied Sciences**, Tamilnadu, v.7, n.9, p.3539-3543, 2018.
- TEBEEST, D.O.; TEMPLETON, G.E.; SMITH JR., R.J. Histopatology of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* on Northern Jointvetch. **Phytopathology**, St Paul, v.68, p.1271-1275, 1978.
- TOWERS, G.H.N.; GIBBS, R.D. Lignin chemistry and the taxonomy of higher plants. **Nature**, London, v. 171, p.25-26, 1953.

- VELOSO, J.S.; CÂMARA, M.P.S.; LIMA, W.G.; MICHEREFF, S.J.; DOYLE, V.P. Why species delimitation matters for fungal ecology: *Colletotrichum* diversity on wild and cultivated cashew in Brazil. **Fungal Biology**, Amsterdam, v.122, p.677-691, 2018.
- VIDAL, M.F. Situação da Cajucultura nordestina após a seca. **Caderno Setorial ETENE** (Escritório Técnico de Estudos Econômicos do Nordeste), v.1, n.4, p.17-25, 2016.
- WEI, Y.D.; BYER, K.N.; GOODWIN, P.H. Hemibiotrophic infection of round-leaved mallow by *Colletotrichum gloeosporioides* f.sp.malvae in relation to leaf senescence and reducing reagents. **Mycology Research**, Manchester, v.101, p.357-364, 1997.
- WHARTON, P.S.; SCHILDER, A.C. Novel infection strategies of *Colletotrichum acutatum* on ripe blueberry fruit. **Plant Pathology**, Wallingford, v.57, p.122-134, 2008.
- WHEELER, M.H.; TOLMSOFF, W.J.; BELL, A.A. Ultrastructural and chemical distinction of melanins formed by *Verticillium dahliae* from (+)-scytalone, 1,8-dihydroxynaphthalene, catechol and L-3,4-dihydroxyphenilalanine. **Canadian Journal Microbiology**, Ottawa, v.24, 289-297, 1998.
- WILSON, C.M. an update on protein stains: amido black, Comassie blue G and Comassie blue R. **Biotechnic & Histochemistry**, Baltimore, v.67, p.224-235, 1992.
- WILSON, U.E.; COFFEY, M.D. Cytological evaluation of general resistance to *Phytophthora infestans* in potato foliage. **Annals of Botany**, London, v.45, p.81-90, 1980.
- WOLF, G.; FRIC', F. A rapid staining method for *Erysiphe graminis* f.sp. *hordei* in and on whole barley leaves with a protein-specific dye. **Phytopathology**, St Paul, v.71, p.596-598, 1981.
- WOOF, B.J.; PIERCE, J.S. Study of protein-polyphenol interaction by differential spectroscopy. **Journal of the Institute of Brewing**, London, v. 74, p.544-549, 1968.
- ZAINUDDIN, R.N.; OMAR, M. Influence of the leaf surface of Hevea on activity of *Colletotrichum gloeosporioides*. **Transactions of the British Mycological Society**, Oxford, v.91, p.427-432, 1988.
- ZAKARIA, M.; BAILEY, J.; PRING, R. Infection processes of *Colletotrichum* isolates from forest trees in the tropics. **Journal of Tropical Forestry Science**, Kuala Lumpur, v.12, p.581-592, 2000.
- ZULFIQAR, M.; BRLANSKY, R.H.; TIMMER, L.W. Infection of flower and vegetative tissues of citrus by *Colletotrichum acutatum* and *C. gloeosporioides*. **Mycology**, College Park, v.88, p.121-128, 1996.