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HISTOPATHOLOGY OF Colletotrichum gloeosporioides ON GUAVA FRUITS (Psidium guajava L.)

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ABSTRACT - Anthracnose, caused by Colletotrichum gloeosporioides, produces brown lesions on guava fruits, causing severe losses on postharvest. In this study, the infection and colonization of guava fruits by C. gloeosporioides has been examined using scanning and transmission electron microscopy. Fruits at the physiologically mature stage were inoculated with a 10⁵ conidia/mL spore suspension. Afterward, fruits were incubated at 25 °C in a wet chamber for periods of 6, 12, 24, 48, 96 and 120 h to allow examination of the infection and colonization process. Conidia germination and appressoria formation occurred six hours after inoculation (h.a.i). Penetration occurred directly via penetration pegs from appressoria, which penetrated the host cuticle 48 h.a.i. Notably, the appressoria did not produce an appressorial cone surrounding the penetration pore. Infection vesicles were found in epidermal cells 96 h.a.i. The same fungal structures were found in epidermal and parenchymal cells of the host 120 h.a.i. Colonization strategy of C. gloeosporioides on guava fruit was intracellular hemibiotrophic.

Index terms: colonization, penetration, pre-penetration, ultrastructure.

HISTOPATOLOGIA DE Colletotrichum gloeosporioides EM FRUTOS DE GOIABEIRA (Psidium guajava L.).

RESUMO - A antracnose, causada por Colletotrichum gloeosporioides, é caracterizada por lesões marrons nos frutos de goiabeira, causando severas perdas em pós-colheita. Os processos de infeção e colonização de C. gloeosporioides foram estudados usando microscópio eletrônico de varredura e transmissão. Frutos em plena maturação fisiológica foram inoculados com suspensão de 10⁵ conídios/mL. Posteriormente, os frutos foram incubados em câmara úmida a 25 °C, por períodos de molhamento de 6; 12; 24; 48; 96 e 120 h para o estudo do processo de infeção e colonização. Germinação de conídios e formação de apressórios ocorreram seis horas após a inoculação (h.a.i). A penetração ocorreu diretamente, por meio da formação do peg de penetração emitido da base do apressório, o qual penetrou na cutícula 48 h.a.i. O apressório não produziu o cone apressorial ao redor do poro de penetração. Foram observadas vesículas de infeção nas células epidérmicas 96 h.a.i. As mesmas estruturas fúngicas foram encontradas nas células epidérmicas e parenquimáticas do hospedeiro, após 120 h da inoculação. A estratégia de colonização de C. gloeosporioides foi do tipo hemibiotrófica intracelular.

Termos para indexação: Colonização, penetração, pré-penetração, ultraestrutura.

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Colletotrichum species cause anthracnose diseases on several plants including cereals, vegetables, garden plants and perennial crops. These pathogens cause severe problems at different stages of crop growth and different parts of the plant like roots, stems, leaves, flowers and fruits (BAILEY; JGER, 1992; JEFFRIES et al., 1990; PRUSKY; FREEMAN; DICKMAN, 2000). Although anthracnose symptoms may appear in the field, depending on the host and environmental conditions, Colletotrichum also cause quiescent infections in fruit, placing them amongst the most important postharvest pathogen. Thereby the pathogen infects the fruit still in the field, but symptoms appear only during ripening (JEFFRIES et al. 1990).

Anthracnose is one of the main guava diseases in all guava-growing countries, causing considerable postharvest losses (LIM; MANICOM, 2003). It is characterized by depressed, soaked, necrotic lesions, with an irregular shape and brown color on the fruit surface. In conditions of high humidity, a salmon-colored conidial mass develops in the center of the lesions. The lesions are also observed in leaves, petioles and branches which do not cause severe damage to the plant but acts as sources of inoculum between seasons (BAILEY; JGER, 1992; PEREIRA; MARTINEZ Jr., 1986; PICCININ et al., 2005).

Colletotrichum species penetrate the host plant through wounds, natural openings or directly via appressorium (JEFFRIES et al., 1990). A large number of Colletotrichum species penetrate directly and the formation of the appressorium is a pre-requisite for the invasion of the host tissues. In contrast, there are species in which the appressorium is optional or unnecessary (EMMETT; PARBERY, 1975).

After penetrating host plant, the pathogen may employ two distinct strategies for colonization: intracellular hemibiotrophy or subcuticular intramural colonization or even a combination of both strategies (BAILEY et al., 1992). Colletotrichum trifolii and C. lindemuthianum adopt the intracellular hemibiotrophy strategy. After penetration, they form a spherical infection vesicle. Hyphae grow from this vesicle and subsequently colonize other host cells (O’CONNELL et al., 1985; MOULD et al., 1991b). In this model, the fungus initially grows biotrophically, without inducing symptoms, but later, in the necrotrophic phase, the fungus induces decay of the host cells causing symptoms (LATUNDE-DADA et al., 1996; O’CONNELL et al., 2000).

Colletotrichum acutatum and C. fragariae in strawberry (CURRY et al., 2002) adopt subcuticular intramural colonization. After penetration, the pathogen grows beneath the cuticle and inside the periclinal and anticalinal wall of the epidermal cells, causing dissolution of the cell wall.

Studies have shown that colonization strategy adopted by C. acutatum depends on the host (CURRY et al., 2002; DIÉGUEZ-URIBEONDO et al., 2005; WHARTON; SCHILDER, 2008), type of vegetal tissue infected (DIÉGUEZ-URIBEONDO et al., 2005; PERES et al., 2005) or host cultivar (WHARTON; SCHILDER, 2008).

The objectives of this study were to characterize the pre-penetration and penetration events of Colletotrichum gloeosporioides on guava fruits, as well as to identify the colonization strategy adopted by this species.

Physiologically mature ‘Kumagai’ guava fruits (Psidium guajava L.) were obtained from Campinas, a guava producer municipality in the State of São Paulo, Brazil. The fruits were disinfested with 0.5% NaOCl for 10 min, rinsed in sterile distilled water, dried at room temperature and conditioned into plastic boxes previously disinfested with 70% ethanol. Inoculation procedures were subsequently carried out on the fruit surfaces.

A pathogenic strain of C. gloeosporioides, obtained from symptomatic guava fruit from commercial field, was cultivated in PDA (Potato-Dextrose-Agar). Later, it was identified via PCR with a specific primer (CgInt) in accordance with methodology described by Mills et al. (1992).

Since the Colletotrichum strain used in the study did not sporulate in any tested culture medium, spores were produced on mycelium-inoculated guava fruits as an alternative. Five millimeters diameter agar plugs were removed from the edges of the colonies grown on PDA and placed on ripe, healthy guava fruit surfaces. Fruits were previously disinfested as described above and arranged inside plastic boxes on top of three sheets of filter paper saturated with water. After eight days, the conidia were collected directly from the diseased fruits by washing the anthracnose lesions with sterile distilled water. This procedure also assured the maintenance of the fungus pathogenicity (WILSON et al., 1990). Suspensions of 10⁴ conidia/mL (as measured with a hemacytometer) were prepared with sterile distilled water and used in all inoculation procedures.

Inoculation was performed by depositing 40 µL of conidial suspension on the surface of fruits. The suspension was deposited in 0.5 cm circles that were previously marked with adhesive plastic tape. Next, the fruits were conditioned in plastic boxes containing three sheets of filter paper saturated with
water and incubated in a humidified atmosphere at 25°C for continuous periods of 6, 12, 24 48, 96 and 120 h. These samples were then used to study the infection and colonization process using scanning (SEM) and transmission electron microscopy (TEM).

Samples of inoculated tissue were removed with a scalpel and transferred to 1.5 mL microtubes containing a modified Karnovsky’s fixative (2.5% glutaraldehyde and 2.5% of formaldehyde solution in sodium-cacodilate buffer 0.05M – pH 7.2 and CaCl₂ 0.001M). These samples were stored at 4°C for at least 48 h and processed for SEM and TEM according to the methods of Bozzaola and Russell (1998).

A portion of the samples that had been inoculated and incubated at 25°C for 24 h were transferred to glass vials and submitted to ultrasonic treatment in a Branson 2210R-DTH (Bransonic, USA) apparatus for 15 minutes after fixation. Subsequently, the samples were processed for visualization by SEM.

For SEM analysis, the samples were removed from the modified Karnovsky’s fixative, rinsed in 0.05 M sodium cacodilate buffer pH 7.2 and postfixed in 1% osmium tetroxide three times every 10 minutes for two hours. Afterward, they were rinsed in distilled water three times of 10 minutes and dehydrated in serial dilutions of pure acetone (30, 50, 70, 90 and 100%), for 10 minutes at each concentration. The procedure was done in triplicate with pure acetone. Samples were then dried to the critical point by transferring the samples to glass vials and submitted to ultrasonic treatment in a CPD 030 critical point dryer (Balzers, Lichtenstein), at 50 mA, for 180 seconds. Observations were made using a Zeiss DSM 940A Scanning Electron Microscope (Carl Zeiss, Germany). Five fruits were analyzed per treatment and the experiment was repeated twice.

For TEM analysis, the samples were removed using gold vapor in a Balzers MED 010 apparatus (Balzers Union, Lichtenstein), at 50 mA, for 180 seconds. Observations were made using a Zeiss EM 900 Transmission Electron Microscope (Carl Zeiss, Germany). The grid was examined in a Zeiss EM 900 Transmission Electron Microscope (Carl Zeiss, Germany). The experiment was performed once, with five replicates per treatment.

It was observed germinated conidia, germ tubes and appressoria adhered to the surface of the inoculated fruits. However, after the ultrasound treatment, only the appressoria remained fixed to the host surface (Figure 1A). The significance of these results lies in the fact that the adhesion of the appressorium plays an important role in the success of the infection. Adhesion of the appressorium ensures that the pathogen remains in contact with the host long enough to originate the penetration peg and subsequently the penetration (BAILEY; JEGER, 1992).

Conidial germination of Colletotrichum is highly variable, starting between three and 48 hours after inoculation (h.a.i) (BAILEY; JEGER, 1992; LOPEZ, 2001). In this study, conidial germination was observed 6 h.a.i, through the formation of the germ tube or appressoria, similar to the findings obtained with C. dematium in cowpea and C. fragariae in cherimoya fruits (SMITH; KORSTEN; AVELING, 1999; VILLANUEVA-ARCE et al., 2006). In coffee plants inoculated with C. gloeosporioides and C. dematium, the conidial germination started 5 and 12 h.a.i, respectively (LINS; ABREU; ALVES, 2007). Conidia of Colletotrichum acutatum germinated 4 h.a. on strawberries (ARROYO et al., 2005) and C. destructivum took 12 h to germinate on tobacco (SHEN; GOODWIN; HSING, 2001).

Colletotrichum gloeosporioides conidia are normally aseptate, however they develop septa during germination (Figure 1B-C) due to mitosis (MOULD et al., 1991a; O’CONNELL et al., 1993).

The germ tube growth was not affected by the presence or location of stomata on the fruit surface (Figure 1D). There was no penetration via the stomata. Penetration occurred directly via penetration pegs from the appressoria. On the other hand C. gloeosporioides did not penetrate directly into the cuticle of mulberry leaf. It penetrates through stomata cavity where an infection vesicle was formed (KUMAR et al., 2001).

The appressoria developed in different positions on the conidia and presented distinct shapes, including spherical, irregular, lobed or clavate (Figure 1E-H). Thus, in this case appressoria morphology
should not be used for species identification.

Ultrastructural analysis showed that the appressoria were surrounded by an extracellular matrix, which appeared to be responsible for the adhesion to the host surface. The area of contact between the appressoria and the fruit surface was relatively narrow (Figure 1 I-K). After the formation of the penetration peg, the cuticle structure remained intact in the region near the pore, supporting the hypothesis that penetration occurs mainly through mechanical force, as reported for *C. acutatum* in strawberry (ARROYO et al., 2005). However, other factors, such as enzyme activation during the process need to be considered (O’CONNELL et al., 1985; KÖLLER, 1991). Cutinase enzyme is present in conidial mucilage and in extracellular fluids of germinated conidia of *Colletotrichum kahawae* in coffee leaves and berries. However, the turgor pressure plays a major role in the coffee cuticle penetration (CHEN et al., 2004).

The appressorial wall is composed of three distinct layers. The third layer was deposited on the appressorium base between the plasmalemma and the inner wall layer (Figure 1 I-K), forming a penetration pore. This layer was adjacent to the wall of the penetration peg, which emerges through the pore and penetrates directly through the cuticle into the epidermal cell wall. Inside the appressorium of some *Colletotrichum* species, such as *C. trifolli* (MOULD et al., 1991a), the infection pore is surrounded by a cone-shaped structure called the appressorial cone. This structure is an extension of the penetration peg wall and may act on the hydrostatic pressure at the penetration spot. However, in this study the appressorial cone was not observed, which is also the case of *C. sublineolum* (WHARTON et al., 2001), *C. truncatum* (O’CONNELL et al., 1993) and *C. acutatum* (ARROYO et al., 2005). The lack of a cone in some species and its presence in others suggests that mechanism of infection by species of *Colletotrichum* depends on the host species.

According to the definition of some authors (BAILEY et al., 1992; PERFECT et al., 1999), the colonization process of *C. gloeosporioides* in guava was intracellular hemibiotrophic, similar to the observed in other species of *Colletotrichum*, such as *C. trifolli* and *C. lindemuthianum* in alfalfa and bean leaves, respectively (O’CONNELL et al., 1985; MOULD et al., 1991a, 1991b). In this study, it was observed at 48 h.a.i that the penetration pegs originated in the base of the appressorium and penetrated directly into the cuticle (Figure 2A). Successful penetration was followed by the formation of structures that are specialized for colonization 96 h.a.i, such as the spherical vesicles inside the cells. The hyphae and vesicles were restricted to the initially infected epidermal cells (Figure 2B). At 120 h.a.i, vesicles and hyphae were found in the epidermal (Figure 2C), and in the parenchymal cells (Figure 2D). Figure 3 summarizes the chronology of events involved in infection and colonization of guava fruit by *Colletotrichum gloeosporioides*.

On the other hand, different colonization strategies were observed by *C. acutatum* and *C. fragariae* in strawberry (CURRY et al., 2002), *C. capsici* in cowpea (PRING et al., 1995) and *C. gloeosporioides* in papaya (CHAU; ALVAREZ, 1983) because these species did not invade the host cells to form a biotrophic relationship with the protoplast. Instead, the infection hyphae grow underneath the cuticle and inside the periclinal and anticlinal wall of the epidermal cells. DIÉGUEZ-URIBEONDO et al. (2005) also reported two different colonization strategies in almonds, such as intracellular hemibiotrophic and subcuticular intramural necrotrophic.

The present study highlights that the infection process and the colonization are independent and specific mechanisms, which vary according to the host-pathogen interaction, corroborating the observations of Emmett and Parbery (1975). Thus, knowledge of specialized infection structures, e.g., germ tubes, appressoria, penetration pegs, infection vesicles, primary hyphae, and secondary hyphae of *Colletotrichum* provides excellent models for studying the cell biology of other plant pathogenic fungi (O’CONNELL et al., 2000).
FIGURE 1 - Scanning and Transmission Electron Microscopy images showing the Colletotrichum gloeosporioides on guava fruits. A. Adhered appressoria after ultrasound treatment (Bar = 5 µm); B. Septa formed in the germinating conidium 6 h.a.i (Bar = 2 µm); C. Septa formed in the germinating conidium 24 h.a.i (Bar = 2 µm); D. Germ tube growth is not directed towards stomata 12 h.a.i (Bar = 10 µm); E. Conidium and sessile spherical appressorium 24 h.a.i (Bar = 2µm); F. Conidium, germ tube and lobed appressoria 24 h.a.i (Bar = 5 µm); G. Conidium with a irregularly-shaped appressorium 24 h.a.i (Bar = 5 µm); H. Conidium and clavate appressorium 24 h.a.i (Bar = 5 µm); I. Appressorium surrounded by extracellular matrix (arrow), 48 h.a.i (Bar = 2 µm); J. Appressorium on the cuticle, 96 h.a.i (Bar = 2 µm); K. Appressorium in contact with the cuticle and the penetration peg originating at its base, 48 h.a.i (Bar = 2 µm). a = appressorium; c =conidium; cu = cuticle; gt = germ tube; p = peg; pp = penetration pore; s = septum; st = stomata; 1st = outer wall layer of the appressoria; 2nd = inner wall layer of the appressoria; 3rd = third layer deposited at the base of the appressoria, between the plasmalemma and the inner wall layer.
FIGURE 2 - Transmission electron microscopy images showing the colonization of *Colletotrichum gloeosporioides* in guava fruits at different times after inoculation. **A.** Penetration peg in the cuticle of the fruit 48 h.a.i (Bar = 2 µm); **B.** Appressorium on the cuticle and the formation of the penetration peg. Vesicle and hypha in the lumen of the epidermal cell, 96 h.a.i (Bar = 5 µm; **C.** Appressorium, penetration peg, vesicle and hypha 120 h.a.i (Bar = 1.7 µm); **D.** Infection hyphae inside the parenchymal cells, 120 h.a.i (Bar = 1.7 µm). a = appressorium; c = conidium; ce = epidermal cell; cp = parenchymal cell; cu = cuticle; h = hypha; p = peg; ve = vesicle.

FIGURE 3 - Chronology of events in the infection and colonization of guava fruit by *Colletotrichum gloeosporioides*.
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