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Prepenetration and Penetration of *Colletotrichum gloeosporioides* into Guava Fruit (*Psidium guajava* L.): Effects of Temperature, Wetness Period and Fruit Age

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Abstract

In this study, we determined the influences of temperature, wetness period and guava fruit age on infection caused by *Colletotrichum gloeosporioides*. Optimal temperatures *in vitro* for germination, appressoria formation and melanization were 22.7, 20.6 and 23°C, respectively. *In vivo*, the optimal temperatures for germination and appressoria formation were 22.5 and 23°C, respectively. Values for germination, appressoria formation and melanization were higher as the wetness period increased. There was no difference in conidial germination and appressorial formation on fruit of different ages. On the surface of 10-, 35- and 60-day-old fruit, despite the high percentage of appressorial formation, there was no development of the penetration peg. Penetration pegs were only observed on the 85- and 110-day-old fruit. Thickness of the cuticle, size and architecture of epidermal and parenchymal cells, as well as the content of phenolic compounds changed as the fruit ripened.

Introduction

Anthracnose is caused by the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., which is a major challenge in guava fruit production worldwide (Lim and Manicom 2003).

This pathogen produces necrotic lesions on fruit and leaves, as well as dead twigs on guava (Lim and Manicom 2003). Therefore, losses due to quiescent infection are proportionally higher once the costs of harvesting, transporting and storing are added to the apparently healthy fruit sold in the market (Swinburne 1983). In guava, the infection process of *Colletotrichum* includes a conidial attachment on the fruit surface, conidial germination, appressorium formation, appressorium melanization and penetration (Moraes et al. 2013). Guidarelli et al. (2011) demonstrated that typical anthracnose lesions developed 3 days postinfection (dpi) on ripe strawberry fruit artificially inoculated with *Colletotrichum acutatum*. On white, immature fruit, the fungus became quiescent and anthracnose lesions started to appear only on 7 dpi, when fruit became red. The authors attributed this difference of susceptibility to the changes in preformed defence mechanisms during fruit ripening.

As fruits ripen, morphologic, biochemical and physiologic changes occur and quiescent infections are activated, promoting the development of the disease (Prusky and Lichter 2007). However, activation of quiescent infection still depends on favourable environmental conditions, such as temperature, which can favour or delay fruit senescence as well as pathogen development (Pandey et al. 1997; Barkai-Golan 2001). Thus, the establishment of the parasitic relationship depends on the dynamic equilibrium between the pathogen, the host and the environment (Jarvis 1994).

Resistance of unripe fruit to pathogen attack can be explained by the absence, presence or insufficient amounts of fungal pathogenicity factors such as pectolytic enzymes; fruit anatomy and thickness of

the cuticle; the presence of antifungal compounds, which differ in composition, number and roles at different stages of fruit ripening; changes in nutrient availability to pathogens (Swinburne 1983; Jeffries et al. 1990; Prusky 1996; Barkai-Golan 2001; Gabler et al. 2003), and the presence of resistance genes that are expressed at different fruit growth stages (Mahasuk et al. 2009, 2013).

The influence of environmental conditions on the pathosystem guava fruit-*C. gloeosporioides* has already been reported in the literature. In India, Pandey et al. 1997 showed that 30°C was the optimal temperature for the development of anthracnose lesions and sporulation on wounded guava fruit. Additionally, disease incidence was higher as the wetness period increased. However, the authors did not investigate the environmental effects on the microscopic events that take place during fruit infection, such as spore germination and appressorial formation. In Brazil, Soares et al. (2008) studied the effect of temperature and wetness period on the development of infection structures of *C. acutatum* and *C. gloeosporioides* isolated from guava. The favourable temperature for conidial germination and appressorial formation ranged from 25 to 30°C under continuous wetness; temperatures in the ranges of 15 to 20 and 35 to 40°C were unfavourable. However, these authors evaluated the fungal behaviour solely *in vitro* and for a shorter period of time than is reported in the present work. In addition, they did not evaluate appressorial melanization, which has been considered a prerequisite for *Colletotrichum* pathogenicity by Kubo and Furusawa (1991). There are distinct environmental requirements for the formation of fungal structures during the different phases of prepenetration, such as those observed by Leandro et al. (2003) for *C. acutatum* on strawberry leaves. Under continuous wetness, these authors showed that the optimal temperatures for conidial germination, appressorial development and secondary conidiation ranged from 23.0 to 27.7, 17.6 to 26.5 and 21.3 to 32.7°C, respectively (Leandro et al. 2003).

The objectives of this study were (i) to identify the optimal environmental conditions for infection of *C. gloeosporioides* on guava fruit based on *in vivo* and *in vitro* experiments; (ii) to determine the effect of guava fruit age on prepenetration and penetration of *C. gloeosporioides*; (iii) to identify the stage of fruit development in which the pathogen becomes quiescent; and (iv) to study morphological and biochemical changes as guava fruit ripens that could be related to the disease development.

Materials and Methods

Inoculum preparation of *C. gloeosporioides*

An isolate of *Colletotrichum* was obtained from diseased fruit in a commercial field in Campinas County in Sao Paulo State, Brazil. A single-spore culture was obtained, which was phylogenetically identified as *C. gloeosporioides* by multilocus sequencing of ITS, GAPDH, ACT, CHS-1, TUB2 and CAL genes (Bragança 2013) according to the method of Weir et al. (2012). The isolate was deposited in the Centraalbureau voor Schimmelcultures (CBS) collection, Utrecht, The Netherlands, under the code CPC20904. Additionally, gene sequences were deposited in the GenBank database under the accession numbers KC566707 (ITS), KC566561 (GAPDH), KC566853 (ACT), KC566275 (CHS-1), KC566131 (TUB2) and KC566420 (CAL).

Discs of 0.5 cm diameter were removed from the edges of the culture on potato dextrose agar (PDA) and placed on 10 healthy, ripe guava fruit surfaces. In addition, 10 non-inoculated healthy fruit were included to check for previously existing quiescent infection. Fruit were arranged in plastic boxes on top of three sheets of filter paper moistened with distilled water to obtain a humidity-saturated environment. After 8 days, the non-inoculated fruit showed no infection, and the conidia were collected from the anthracnose lesions on the inoculated fruit by washing the lesions with sterile distilled water. A spore suspension was prepared, and its concentration was adjusted to 10^5 conidia/ml. This same concentration was used in all inoculation procedures.

Evaluation of the effects of temperature and wetness duration on prepenetration of *C. gloeosporioides in vitro*

Droplets of 40 μl of the conidial suspension were deposited on the surface of polystyrene Petri dishes that were maintained in closed plastic boxes ($L \times W \times H = 11 \times 11 \times 3.5$ cm) on top of three sheets of filter paper saturated with water to keep the humidity high. The plastic boxes were kept in growth chambers (Conviron, Winnipeg, Canada) at 10, 15, 20, 25, 30, 35 and 40°C and 6, 12, 24, 36 and 48 h of wetness duration in continuous light. At the end of each wetness period, conidial germination was interrupted by adding 15 μl of lactoglycerol followed by placing cover slips on the droplets.

Subsequently, the Petri dishes were placed on the stage of a light microscope (Zeiss Axioskop 2; Zeiss, Jena, Germany), and conidial germination, appressorial development and melanization were assessed at

400× magnification. One hundred conidia per replication were evaluated. Conidia were considered to be germinated when the germination tube exceeded half of the diameter of the conidia. The experiment was performed twice, with six replications per treatment. Each droplet corresponded to one replication.

Evaluation of the effects of temperature and wetness duration on prepenetration events of *C. gloeosporioides in vivo*

Physiologically mature 'Kumagai' guava fruit were obtained from a Campinas County producer. Fruit were disinfested with 0.5% sodium hypochlorite for 10 min, rinsed in distilled water, dried at room temperature and conditioned in plastic boxes, previously disinfested with ethanol 70%. Each treatment contained five inoculated fruit and five non-inoculated fruit, arranged on top of three sheets of filter paper saturated with water. Inoculation was performed by depositing droplets of 40 μl of the conidial suspension on the surface of the fruit in 0.5 cm diameter circles, previously marked with adhesive plastic tape. The non-inoculated fruit were included to check for quiescent infection that could have been present previously on the fruit. The boxes were kept in a growth chamber (Conviron) in continuous light at 10, 15, 20, 25 and 30°C and 6, 12 and 24 h of wetness duration.

After these periods, the non-inoculated fruit showed no infection, and samples of inoculated tissue were removed with a scalpel and processed for scanning electron microscopy (SEM) according to the methodology described by Bozzola and Russel (1998). Inoculated samples (0.5 cm in diameter and 2–3 mm in thickness) were fixed with modified Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.001 M $CaCl_2$ and 0.05 M cacodylate buffer, pH 7.2) for 48 h. Samples were subsequently rinsed three times for 10 min each in 0.05 M sodium cacodylate buffer and postfixed in a 1% osmium tetroxide solution for 2 h. Samples were then immersed in distilled water three times for 10 min each and dehydrated with acetone in increasing concentrations (30, 50, 70, 90 and 100%) for 10 min per concentration. The procedure was performed in triplicate with pure acetone. Samples were dried to the critical point by immersing fragments in liquid carbon dioxide in a CPD 030 critical point dryer (Bal-Tec, Balzers, Liechtenstein), then fixed in aluminium stubs with double-sided carbon tape. The stubs with the samples were metalized by gold vapour in a MED 010 sputtering machine (Bal-Tec) at 50 mA for 180 s. Observations were made using a Zeiss DSM 940A Scanning Electron Microscope (Carl Zeiss, Oberkochen, Germany), and the images were collected as electronic files.

One hundred conidia per replicate were evaluated, and the percentages of germination and appressorial development were calculated. The experiment was performed twice, with five replicates per treatment.

Evaluation of prepenetration and penetration stages of *C. gloeosporioides* on guava fruit of different ages

'Kumagai' guava fruit of 10, 35, 60, 85 and 110 days after petal drop (approximate diameters of 1, 2, 3, 5 and 7 cm, respectively) were obtained from a Campinas County producer. After fruit disinfestation, droplets of 40 μl of the conidial suspension were deposited on the surface of each fruit, which were placed in plastic boxes as previously described and kept in a growth chamber at 25°C. After 48 h, samples were excised with a scalpel and processed for visualization in a scanning electron microscope, following the procedures previously described.

For transmission electron microscopy (TEM) analyses, the samples were processed as described for SEM procedures up to the postfixation in osmium tetroxide 1% for 2 h. Afterwards, these samples were cut into pieces of approximately 0.1 × 0.3 cm and 'en bloc' contrasted with 0.5% uranyl acetate overnight. Subsequently, they were dehydrated in increasing concentrations of acetone (30, 50, 70, 90 and 100%) for 10 min at each concentration. The procedure was performed in triplicate with pure acetone. Afterwards, the samples were embedded in Spurr's low-viscosity epoxy resin and polymerized for 72 h at 70°C. The blocks were trimmed in Leica EM TRIM equipment (Leica, Vienna, Austria) and cut in a Leica Ultracut UCT microtome (Leica).

Ultra-fine sections (60–70 nm) were obtained with a Diatome 45° diamond knife (Diatome, Biel, Switzerland) and collected in Formvar-coated 100 mesh copper grids. After staining with uranyl acetate (3%) and lead citrate (Reynolds 1963) for 15 min, each grid was examined in a Zeiss EM 900 Transmission Electron Microscope (Carl Zeiss).

Morphological and biochemical changes in guava fruit with different ages

Surface samples (0.5 cm in diameter and 2–3 mm in thickness) were excised from 'Kumagai' guava fruit of 10, 35, 60, 85 and 110 days after petal drop and fixed with modified Karnovsky's fixative for 48 h. They were then dehydrated in solutions of ethanol with increasing concentrations (30, 50, 70, 90 and 100%)

for 10 min at each concentration. The procedure was performed in triplicate with pure ethanol. The samples were embedded in acrylic resin and pure ethanol (1 : 1) for 5 h. Samples were then embedded in pure resin and kept overnight at room temperature (Ruetze and Schmitt 1986). The polymerized blocks were cut in a microtome (Spencer Lens Co., Buffalo, NY, USA) into sections 5 μm thick and were immediately placed on histological glass slides and stained.

Astrablue-Chrysoidin-New Fuchsin (ACN) stain was used to visualize structures of the fruit tissues and phenolic compounds (Neumüller and Hartmann 2008). The slides were immersed in ACN for 5 min, rinsed in running water and dried at room temperature. Observations were made using a light microscope (Zeiss Axioskop 2; Zeiss). Cuticle thickness of guava fruit with different ages was also analysed using a TEM. These samples were processed as described previously.

Data analysis

Data regarding conidial germination, appressorial development and appressorial melanization influenced by temperature and wetness duration were analysed using nonlinear regression. For the *in vitro* experiments, the beta-monomolecular model proposed by Hau and Kranz (1990), described by the equation

$$Y = [B1 \times ((T - B2)^{B3}) \times ((B4 - T)^{B5})] \times [B6 \times (1 - B7 \times \exp(-B8 \times W))],$$

was adjusted to the data, using the STATISTIC PROGRAM version 6.0 (StatSoft, Tulsa, OK, USA), in which Y represents the variable analysed; T represents temperature; W represents wetness duration; $B2$ and $B4$ represent minimum and maximum temperatures, respectively; $B1$, $B3$, $B6$ and $B7$ are equation parameters with no biological significance; $B5$ represents amplitude of the curve asymptotic range; and $B8$ is related to the increase of Y as a function of the wetness duration.

For the *in vivo* experiments, data were analysed through nonlinear regressions, using the logistic procedures of the SAS program for the model:

$$Y = \exp(\beta)/1 + \exp(\beta) \times 100,$$

where Y represents the variable analysed and β is the linear predictor that corresponds to the combination of parameters T (temperature), W (wetness) and P (parameters of the equation). In all cases, these variables were represented by: $(P0 + P1 \times W + P2 \times W^2 + P3 \times T + P4 \times T^2)$.

The adequacy of the models for the data (Figs 1 and 2) was analysed by the coefficients of determination (R^2) obtained from nonlinear regression. For each experiment, an adjustment was made and all parameters of each experiment were compared by replication using a t-test (Campbell and Madden 1990). Because there was no statistically significant difference between them (P = 0.05), a new adjustment was obtained using data from both experiments.

For the experiment performed with guava fruit of different ages, the statistical analysis was performed in the SAS program using the general linear models (GLM) procedure.

Results

Evaluation of the effects of temperature and wetness duration on prepenetration of *Colletotrichum gloeosporioides in vitro*

As temperature and wetness duration increased, percentages of conidial germination, appressorial development and appressorial melanization also gradually increased, as observed in the adjustment of the beta-monomolecular model (Table 1). The response surface to estimate conidial germination (Fig. 1a) was described by:

$$Y = [0.653 \times ((T - 4.760)^{0,76}) \times ((40 - T)^{0,74})] \times [1.636 \times (1 - 0.795 \exp(-0.064 \times W))].$$

The appressorial development (Fig. 1b) was described by:

$$Y = [0.0002 \times ((T - 4.032)^{1.81}) \times ((40.869 - T)^{2.23}))] \times [(2.805 \times (1 - 0.996 \times \exp(-0.079 \times W))].$$

The appressorial melanization (Fig. 1c) was described by:

$$Y = [0.002 \times ((T - 6.122)^{1.42}) \times ((40 - T)^{1.43}))] \times [(14.204 \times (1 - 1.499 \times \exp(-0.081 \times W))].$$

The variables analysed reached the maximum between 20 and 25°C in all the wetness periods tested. At the 48 h-wetness period, the optimal temperatures were 22.7°C for conidial germination, 20.6°C for appressorial development and 23.0°C for appressorial

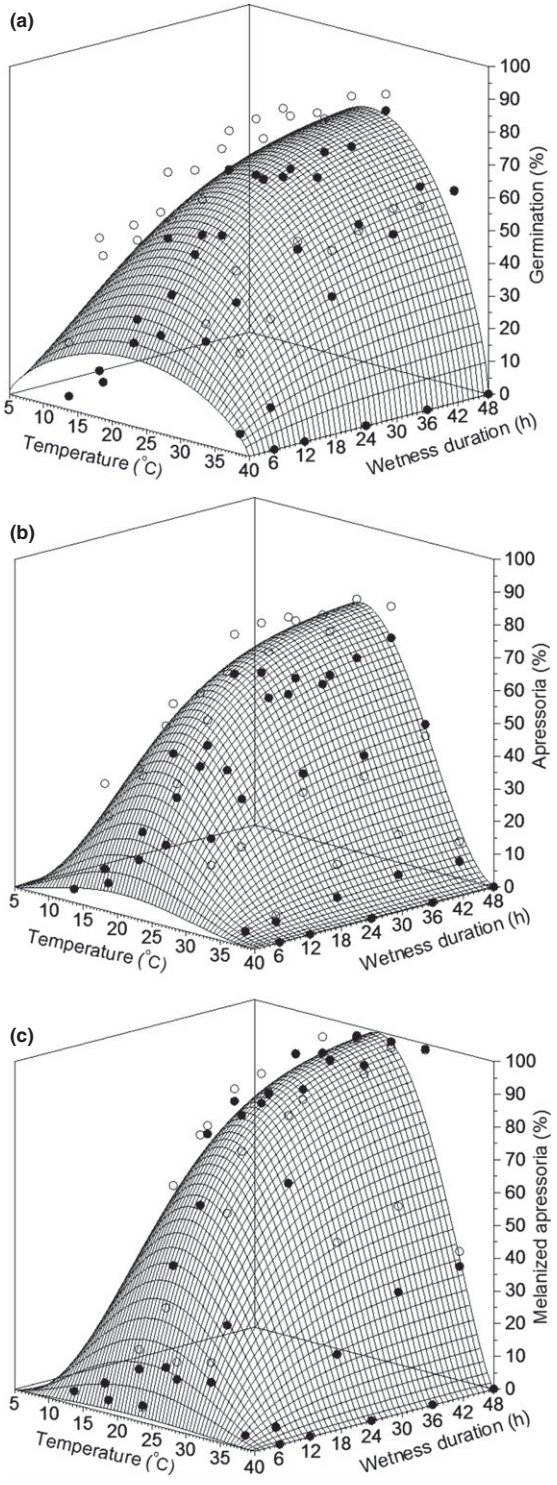

Fig. 1 Response surfaces describing the *in vitro* relationship of temperature and wetness duration on germination (a), appressorial development (b) and appressoria melanization (c) of *Colletotrichum gloeosporioides*. White dots = results from the first experiment; black dots = results from the second experiment.

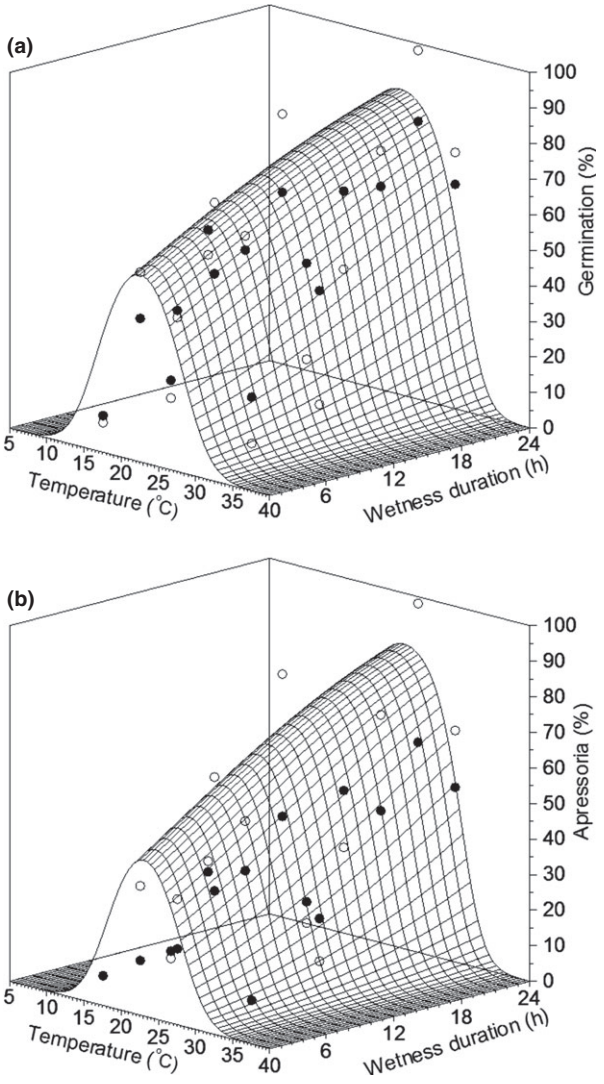

Fig. 2 Response surfaces describing the relationship of temperature and wetness duration on germination (a) and appressorial development (b) of *Colletotrichum gloeosporioides* on guava fruit surfaces. White dots = results from the first experiment; black dots = results from the second experiment.

melanization. At these temperatures, conidial germination corresponded to 78%, appressorial development to 76% and appressorial melanization to 98%. At 40°C, there was no conidial germination.

Conidial germination, appressorial development and appressorial melanization reached the maximum at the 48-h wetness period. At 25°C, conidial germination at 6, 24 and 48 h of wetness duration was 37, 66 and 77%, respectively. At 25°C and at 6, 24 and 48 h of wetness duration, appressorial development was 26, 59 and 68%, and appressorial melanization was 8, 78 and 97%, respectively.

Table 1 Estimated parameter values (*B*1–*B*8) and coefficients of determination (R^2) from the surface response equation describing the *in vitro* relationship of temperature and wetness period on the prepenetration stages of *Colletotrichum gloeosporioides*

Prepenetration stages (*Y*)	*B*1[a]	*B*2[a]	*B*3[a]	*B*4[a]	*B*5[a]	*B*6[a]	*B*7[a]	*B*8[a]	R^2
Germination	0.65	4.76	0.76	40.00	0.74	1.63	0.79	0.06	0.88
Appressoria	2×10^{-4}	4.03	1.81	40.86	2.23	2.80	0.99	0.07	0.91
Appressoria melanization	2×10^{-3}	6.12	1.42	40.00	1.43	14.20	1.49	0.08	0.90

[a]*B*2 and *B*4 represent minimum and maximum temperatures, respectively; *B*1, *B*3, *B*5, *B*6 and *B*7 are equation parameters with no biological significance; *B*8 is related to the increase of prepenetration stages as a function of the wetness duration.

Evaluation of the effects of temperature and wetness duration on prepenetration events of *Colletotrichum gloeosporioides in vivo*

Conidial germination and appressorial development occurred at all tested temperatures *in vivo* and were higher as the wetness period increased (Fig. 2, Table 2).

The surface response for conidial germination (Fig. 2a) was described by:

$$Y = [\exp(-16.135 + (0.074 \times W) + (1.434 \times T) + (-0.031 \times T^2))]/[1 + \exp(-6.135 + (0.074 \times W) + (1.434 \times T) + (-0.031 \times T^2))] \times 100.$$

The appressorial development (Fig. 2b) was described by:

$$Y = [\exp(-20.004 + (0.088 \times W) + (1.706 \times T) + (-0.037 \times T^2))]/[1 + \exp(-20.004 + (0.088 \times W) + (1.706 \times T) + (-0.037 \times T^2))] \times 100.$$

According to the models, the temperature range of 20–25°C resulted in the highest percentages of conidial germination and appressorial development in all wetness periods tested. Optimal temperatures for conidial germination and appressorial development, at 24 h of wetness period, were 22.5 and 23°C, respectively. At these temperatures (22.5 and 23°C), conidia germinated and appressoria developed at rates of 86 and 85%, respectively. At 10°C, the percentage of conidial germination and appressorial formation were lower than 5%, and at 30°C, these variables were lower than 50%, in all wetness durations tested.

The results of the influence of wetness period *in vivo* at 25°C, conidial germination and appressorial development increased from 57 and 51% with 6 h of wetness period to 67 and 64% with 12 h and to 83 and 82% with 24 h of wetness duration, respectively.

Table 2 Estimated parameter values (*P*0–*P*4) and coefficients of determination (R^2) from the surface response equation describing the *in vivo* relationship of temperature and wetness period on the prepenetration stages of *Colletotrichum gloeosporioides*

Prepenetration stages (*Y*)	*P*0[a]	*P*1[a]	*P*3[a]	*P*4[a]	R^2
Germination	−16.135	0.074	1.434	−0.031	0.73
Appressoria	−20.004	0.088	1.706	−0.037	0.79

[a]*P*0, *P*1, *P*2, *P*3 and *P*4 represent equation parameters with no biological significance.

Evaluation of prepenetration and penetration events of *C. gloeosporioides* in guava fruit of different ages

The influence of different ages of guava fruit, quantified using SEM, was not significant for conidial germination (P = 0.245) or appressorial development (P = 0.306). High percentages of these variables were verified in all fruit ages (Fig. 3).

TEM observations showed direct penetration of the pathogen into the fruit cuticle through the penetration peg, developed at the appressorium base. Penetration pegs were verified only on fruit that was 85 and 110 days old and were restricted to the cuticle, not reaching the epidermal cells (Fig. 4b,c). In 60-day-old fruit, no penetration pegs were observed; however, a pore on the appressorium cell wall, through which the penetration peg would most likely be emitted (Fig. 4a), was observed. Extracellular adhesive material was always present around the appressoria for all fruit ages. On the surface of 10- and 35-day-old fruit, no appressoria with penetration pegs were observed.

Morphological and biochemical changes in guava fruit with different ages

Biochemical and morphological changes were observed in fruits as they aged. ACN staining showed phenolic compounds in the cells of the epidermis and parenchyma. These compounds were identified by the orange colour inside cell vacuoles (Neumüller and Hartmann 2008). As the fruit aged, there was a

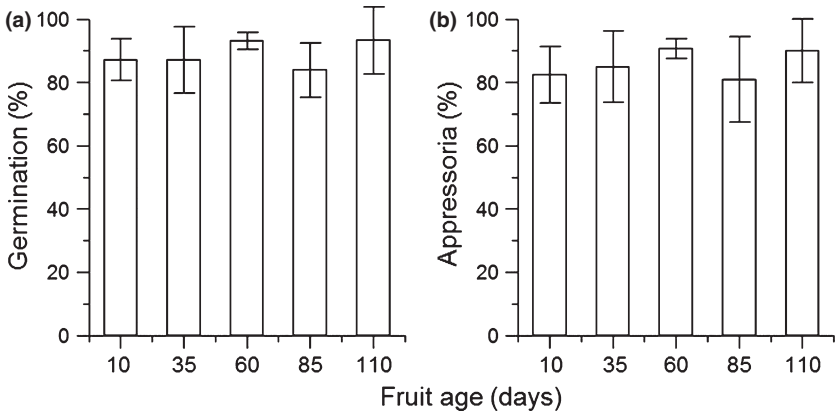

Fig. 3 Germination (a) and appressorial development (b) of *Colletotrichum gloeosporioides* on the surface of guava fruits with different ages after 48 h of wetness duration at 25°C. Bars represent standard errors of the mean.

gradual reduction in the amounts of these compounds (Fig. 5).

Regarding the morphological changes, in the 10-day-old fruit, there were fewer intercellular spaces, higher numbers of secreting structures (lysigenous cavities), and a higher amount of sclereids (petrea cells) observed when compared with older fruit. Tissues of the 35- and 60-day-old fruit showed anatomy similar to the 10 day-old fruit. However, a slight decline in the number of lysigenous cavities and sclereids as well as an increase in cell size and intercellular spaces was observed. Greater histological changes were observed in tissues of the 85- and 110-day-old fruit. These tissues showed much larger cells and wide intercellular spaces. The lysigenous cavities were not found. In addition, cells in the parenchyma expanded radially around the sclereids. A similar result was found for cuticle thickness. The cuticle thickness was gradually reduced with the increase of the age of fruits (Fig. 6).

Discussion

This study reports results of the influence of temperature, wetness duration and fruit age in the prepenetration and penetration of *C. gloeosporioides* on guava fruit. Conidial germination, appressorial formation and melanization occurred in a wide range of temperatures, from 10 to 35°C, under continuous wetness. Optimal temperatures for conidial germination and appressorial development were 22.7 and 20.6°C *in vitro* (48 h of wetness), and 22.5 and 23°C *in vivo* (24 h of wetness), respectively. These results lay the groundwork for a disease prediction model for anthracnose on guava fruit, similar to that suggested by Miles et al. (2013) for the pathosystem *C. acutatum*-blueberry. Similar

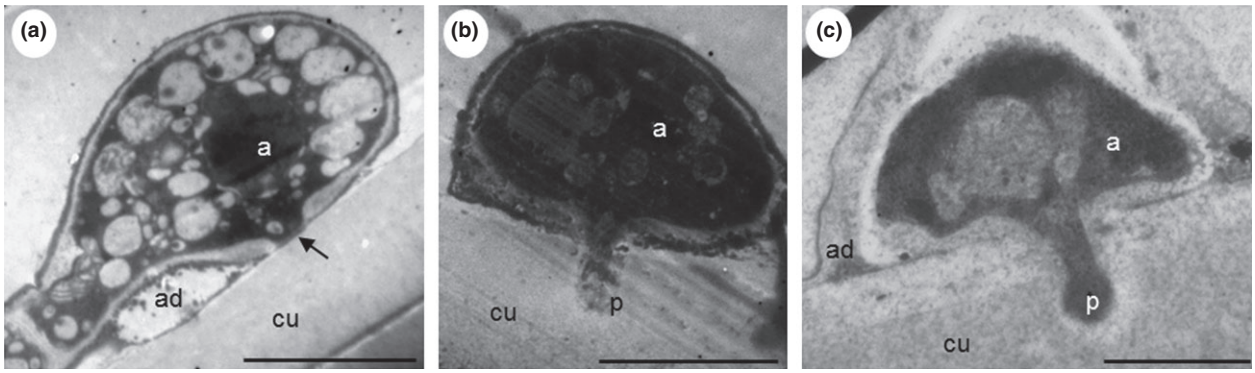

Fig. 4 Transmission electron microscopy photomicrographs showing penetration of *Colletotrichum gloeosporioides* on guava fruit with different ages. Cuticle of 60-day-old fruit showing appressorium with a pore (arrow) through which the penetration peg is emitted (Bar = 1 μm) (a); Penetration peg on 85-day-old fruit (Bar = 2 μm) (b); Penetration peg restricted to the cuticle on 110-day-old fruit (Bar = 2 μm) (c). a, appressorium; cu, cuticle; ec, epidermal cell; p, penetration peg; ad, adhesive material.

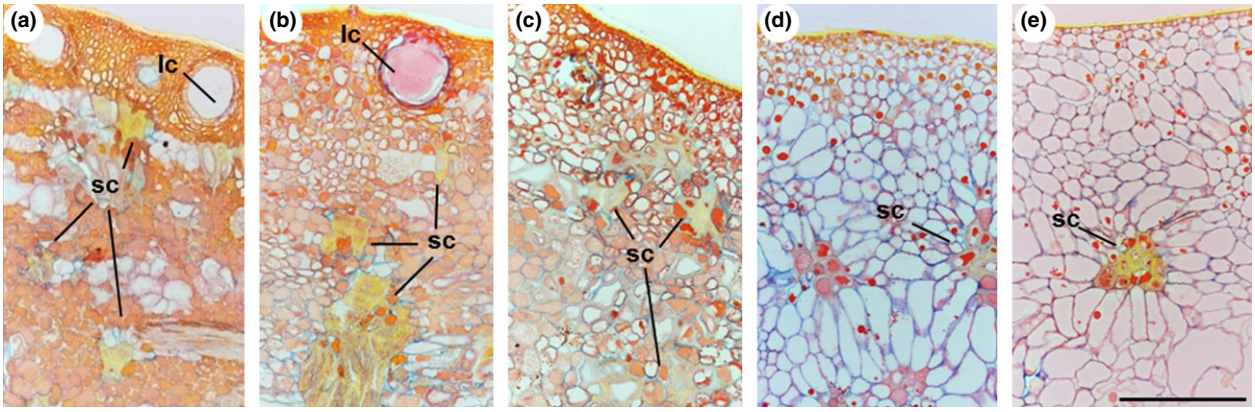

Fig. 5 Light microscopy showing the anatomy and chemical changes of guava fruits with different ages. Fruit ages are 10, 35, 60, 85 and 110 days in a–e, respectively. Sections stained with Astrablue-Chrysoidin-New Fuchsin ('ACN'). Phenolic contents (revealed by the orange colour in the tissues) decrease as fruit age. (Bar = 100 μm). lc, lysigenous cavity; sc, sclereids.

behaviour was reported by Soares et al. (2008) when comparing isolates of *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*, isolated from guava fruit. For *C. gloeosporioides*, these authors reported optimal germination between 20 and 25°C after 6 h of wetness duration *in vitro*. At 10 and 35°C, germination and appressorial development were lower than 10%.

The optimal temperature for appressorial melanization was 23°C, and the increase in appressorial melanization was more evident as the wetness duration increased, suggesting that this variable is more dependent on the water on the host surface. Melanin biosynthesis is a prerequisite for successful invasion of the plant host by appressoria of *Colletotrichum* (Kubo and Furusawa 1991; Kubo 2005). The general infection sequence of *C. lagenarium* (Ge and Guest 2011), *C. truncatum* (Ranathunge et al. 2012) and *C. acutatum* (Wharton and Schilder 2008) is conidial attachment, conidial germination and formation of melanized appressoria, penetration through the penetration peg, formation of infection vesicles, primary hyphae and necrotrophic secondary hyphae.

Optimal temperatures for conidial germination were similar to those observed for appressorial formation for *in vitro* and *in vivo* experiments. This suggests that changes in temperature are unnecessary during these two stages of *C. gloeosporioides* prepenetration in guava fruit. Changes in temperature between these two stages are not rare and have been reported in previous studies. Leandro et al. (2003) reported that conidial germination of *C. acutatum* on strawberry occurred in a narrower range of temperatures (23–27.7°C) than that observed for appressorial development (17.6–26.5°C).

Conidial germination and appressorial development showed slightly higher rates *in vivo* when compared with the *in vitro* experiments (Figs 1 and 2). This behaviour may have been influenced by exogenous compounds present on the guava fruit surface. On avocado fruit, it was also observed that conidial germination and appressorial development of *C. gloeosporioides* were higher on fruit, in this case due to the surface wax that acted as a chemical promoter (Podila et al. 1993; Flaishman et al. 1995). In addition to chemical composition, the topography of the fruit surface can also influence prepenetration events (Kolattukudy et al. 1995).

In this work, no difference was observed in conidial germination and appressorial development in fruit of different ages. These results are in agreement with those obtained for nectarine and plum fruit inoculated with *Botrytis cinerea* (Fourie and Holz 1995). However, in guava fruit inoculated with *Guignardia psidii*, conidial germination and appressorial formation in 110-day-old fruit were five and eight times higher, respectively, than observed in 10-day-old fruit. (Escanferla et al. 2009).

Unlike conidial germination and appressorial development, the penetration was influenced by the fruit age. We observed penetration pegs in 85- and 110-day-old fruit. Successful penetration of *C. gloeosporioides* on guava fruit is followed by the formation of spherical vesicles inside the cells 96 h after inoculation (Moraes et al. 2013). Penetration may be correlated with the thickness of the cuticle because the cuticle thickness declined as the fruit aged, as observed with the electron microscope. For example, during the development of grape berries, modifications in thickness and density of the cuticle were

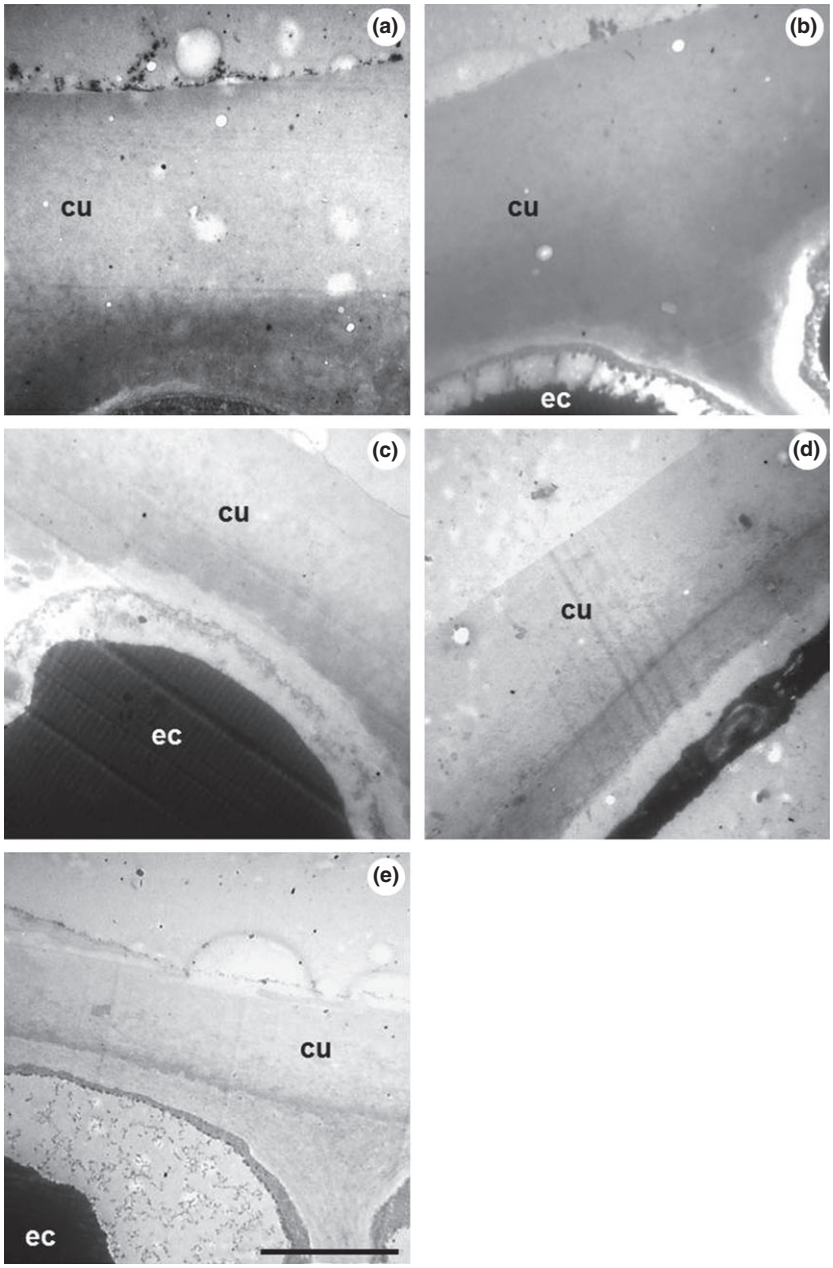

Fig. 6 Transmission electron microscopy photomicrographs showing the cuticle changes in thickness for guava fruit with different ages: 10, 35, 60, 85 and 110 days old in a–e, respectively. (Bar = 10 μm). ec, epidermal cell; cu, cuticle.

correlated with grape susceptibility to bunch rot disease (Comménil et al. 1997). In addition to the cuticle thickness, Gabler et al. (2003) also correlated the epidermal cell number and the thickness of epidermal cells to the susceptibility of grey mould caused by *Botrytis cinerea*. In strawberries, the incidence and severity of the anthracnose was higher in ripe compared with immature fruit (Wilson et al. 1990; Tanaka et al. 1994). Guidarelli et al. (2011) observed that early interaction events were similar on unripe and ripe strawberry fruit. Therefore, 24 h postinfection, on unripe fruit, *C. acutatum* became quiescent as melanized appressoria, but on ripe fruit, it displayed subcuticular necrotrophic invasion. These authors suggest that several genes are regulated differently in the host during ripening or upon pathogen challenge, which can explain the production of quiescence-related compounds or specific pathogen-induced responses.

Pathogen penetration in fruit close to physiologic maturation may also be correlated with high levels of antifungal compounds in unripe fruit tissues. In this study, we observed that phenolic compounds gradually reduced as the fruit aged, suggesting that these compounds found on the fruit surface also contribute to discontinuation of the infection process of *C. gloeosporioides*. Preformed phenolic compounds have been found to inhibit the development of *Botrytis cinerea* in immature strawberries (Terry et al. 2004). Unripe avocado fruit were resistant to *C. gloeosporioides* due to the high concentration of the phenolic compound, diene (1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene), which declined as fruit ripened (Prusky et al. 1991).

Decreasing tannin content, which is found in high amounts in unripe guava fruit, may be related to the susceptibility of ripe fruit to *C. gloeosporioides* (Selvaraj et al. 1999). In unripe banana fruit, the tannin inactivated extracellular enzymes produced by fungal pathogens, reducing infection (Greene and Morales 1967). The susceptibility of fruit to diseases may also be related to the increase in the concentration of sugars, polyphenols and ethylene and the reduction of cell wall constituents, such as cellulose, hemicelluloses and lignin, as guava fruit ripen (Prusky 1996; Bulk et al. 1997; Jain et al. 2003).

It can be concluded that in the pathosystem, guava fruit-*C. gloeosporioides*, there are physical and biochemical mechanisms that act simultaneously or sequentially in pathogen development and quiescence. Other mechanisms may be present; therefore, further investigations are required to elucidate them.

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