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PLANT PROTECTION - Article

Alkaloids and phenolics biosynthesis increases mango resistance to infection by *Ceratocystis fimbriata*

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ABSTRACT: Mango wilt, caused by *Ceratocystis fimbriata*, is one of the most important diseases affecting mango yields in Brazil. Information regarding the biochemical mechanisms involved in mango resistance against *C. fimbriata* is absent in the literature. Thus, the present study determined and quantified alkaloids and phenolics in the stem tissue of mango plants from Palmer (susceptible) and Ubá (resistant) cultivars. Furthermore, it was examined the effect of these secondary metabolites against *C. fimbriata* growth *in vitro*. The high-performance liquid chromatography revealed that the concentration of two alkaloids (theobromine and 7-methylxanthine) and six phenolic compounds (caffeic acid, *p*-coumaric acid, gallic acid, protocatechuic acid, catechin and epicatechin) in the inoculated plants from cv. Ubá was higher in comparison with inoculated plants from cv. Palmer. The concentration of the secondary metabolites was higher in the non-inoculated plants from cv. Palmer than in the inoculated ones, while the opposite was observed for plants of cv. Ubá. Peaks in the concentrations of secondary metabolites in the inoculated plants from both cultivars occurred at 7 and 14 days after inoculation. The different concentrations (10 to 30 mg·mL^{-1}) of secondary metabolites added to the Petri dishes greatly inhibited *C. fimbriata* growth over time. These results suggest that secondary metabolites played an important role in the resistance of mango plants against *C. fimbriata* infection.

Key words: *Ceratocystis fimbriata*, *Mangifera indica*, high-performance liquid chromatography, host defense responses, secondary metabolism in plants, vascular pathogen.

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most important tropical fruit crops cultivated worldwide (FAO 2014). Among the factors that greatly improve mango quality and yield are the adaptability of the cultivars to different environmental conditions and their resistance to multiple diseases (Carvalho et al. 2004). Mango wilt caused by the fungus *Ceratocystis fimbriata* Ellis Halst. (Ferreira et al. 2010; Ribeiro 2005; Van Wyk et al. 2007; Viégas 1960) induces the death of the entire tree, either a few months after the fungus penetrates the roots or more slowly if it enters through wounded branches of the canopy, which is therefore a serious concern for growers (Ribeiro 2005; Van Wyk et al. 2007).

The acquisition of certified samplings free of *C. fimbriata* from suitable nursery and the eradication of mango trees with disease symptoms are some of the strategies employed by growers to reduce the yield losses caused by mango wilt (Ribeiro 2005; Van Wyk et al. 2007). In Brazil, the use of mango cultivars showing non-race specific resistance to *C. fimbriata* infection is the most effective strategy adopted by growers to control mango wilt, mainly because chemical control is not efficient (Carvalho et al. 2004; Ribeiro 2005; Rossetto et al. 1996).

Generally, the resistance of trees against infection by pathogens occurs at the biochemical level through the participation of pre- and/or post-production of antimicrobial compounds (Nicholson and Hammerschmidt 1992). Primary →

metabolites are those produced by all plant cells that are directly involved in growth, development or reproduction, while the secondary metabolites are commonly involved in host defense response (Freeman and Beattie 2008; Mazid et al. 2011). Secondary metabolites are classified as alkaloids, terpenoids, compounds containing sulphur and phenolics according to the biosynthetic pathways (Freeman and Beattie 2008; Mazid et al. 2011).

Alkaloids are a large class of nitrogenous compounds found in many vascular plants; alkaloids include caffeine (1,3,7-trimethylxanthine), cocaine, morphine, nicotine and theobromine and can exhibit antimicrobial activity against pathogens (Freeman and Beattie 2008; Garba and Okeniyi 2012; Kim and Sano 2008; Mazid et al. 2011; Scarpari et al. 2005). Araujo et al. (2015) observed that preventive spray of mango plants with acibenzolar-S-methyl and potassium phosphite reduced mango wilt development, induced microscopic defense responses to infection by *C. fimbriata* and potentiated the synthesis of the phenolics compounds and alkaloids such as theobromine and 7-methylxanthine.

Terpenes constitute the largest class of secondary metabolites and are united by their common biosynthetic origin from acetyl-coA or glycolytic intermediates (Freeman and Beattie 2008; Mazid et al. 2011). A vast majority of the different terpenes structures produced by plants are presumed to be involved in defense (Freeman and Beattie 2008; Mazid et al. 2011). According to Hall et al. (2011), the accumulation of amorphous materials identified as terpenoids, i.e. hemigossypol, desoxyhemigossypol, desoxymethoxyhemigossypol and gossypol in the xylem vessels and adjacent parenchyma cells in the roots of a resistant cotton cultivar, restricted the colonization of *Fusarium oxysporum* f. sp. *vasinfectum*, a vascular pathogen.

Secondary metabolites containing sulphur (S) includes glucosinolates, glutathione, phytoalexins, thionins, defensins and allinin, which have been linked with the defense of plants against plant pathogens (Mazid et al. 2011). Williams et al. (2002) reported the presence of sulfate, glutathione, and cysteine in the cells of the stem tissue of a resistant variety of tomato in response to infection by *Verticillium dahlia* (vascular pathogen). X-ray microanalysis also revealed a higher accumulation of S in xylem parenchyma cells, vessel walls, vascular gels, and tyloses, structures in potential contact with hyphae and linked with defense to *V. dahlia* (Williams et al. 2002). Araujo et al. (2014b), using X-ray microanalysis, revealed that the tissues of Ubá cultivar (resistance mango

wilt) had higher levels of insoluble sulfur than those of Haden cultivar (susceptible).

Phenolics are produced primarily via shikimic acid and malonic acid pathways in plants (Freeman and Beattie 2008; Nicholson and Hammerschmidt 1992). Flavonoids (apigenin, catechin, epicatechin, kaempferol, luteolin, myricetin, naringin and quercetin), hydroxybenzoic acids (gallic acid, protocatechuic acid, salicylic acid, syringic acid and vanillic acid) and hydroxycinnamic acids (coumaric acid, caffeic acid, ferulic acid, *p*-coumaric acid and sinapic acid) are among the most important phenolics produced by plants to cope with infection by pathogens (Cushnie and Lamb 2005; Dixon and Paiva 1995; Nicholson and Hammerschmidt 1992). Phenolics that are constitutively present or that are produced during the infection process of a certain pathogen play a pivotal role in the host defense (Nicholson and Hammerschmidt 1992), primarily because of their effect on cell wall lignification (Benhamou and Bélanger 1998), antimicrobial activity (Cushnie and Lamb 2005; Rodrigues et al. 2003), modulation of plant hormones involved in defense signaling pathways and scavenging of reactive oxygen species (Dixon and Paiva 1995). Prusky and Keen (1993) reported the involvement of preformed antifungal compounds such as the resorcinol (phenolic lipids) in the resistance of avocado and mango fruits to fungal decay. Rodrigues et al. (2003) investigated the ultrastructural properties of the rice-*Pyricularia oryzae* interaction after Si treatment and observed an abundant occurrence of empty fungal hyphae surrounded by phenolic-like compounds that were further identified as momilactones A and B (Rodrigues et al. 2004). In leaves and fruits of many mango cultivars, several antimicrobial alkaloids and phenolic compounds have been identified (Ajila et al. 2010; Garba and Okeniyi 2012; Kaur et al. 2010; Prusky and Keen 1993; Ribeiro et al. 2008), but to the best of our knowledge, they were never associated with basal or constitutive resistance against mango wilt.

At the microscopic level, Araujo et al. (2014a,b) produced novel evidences that phenolic-like compounds were involved in the resistance of mango cultivars against *C. fimbriata* infection. To advance our knowledge of the biochemical mechanisms involved in mango resistance against *C. fimbriata*, the present study determined and quantified alkaloids and phenolics in the stem tissue of mango plants from cultivars Palmer and Ubá, which are susceptible and resistant (Araujo et al. 2014a,b), respectively, to mango wilt. Furthermore, it was examined the effect of these secondary metabolites against *C. fimbriata in vitro*. →

MATERIALS AND METHODS
Plant material

Mango plants from cultivars Palmer (susceptible) and Ubá (resistant) (scions) were obtained from a commercial nursery (Dona Euzébia city, Minas Gerais State, Brazil), and their rootstock was from the cv. Imbú. The 1.5-year-old plants were transplanted into plastic pots containing 8 kg of soil, sand and manure in a 2:1:1 proportion. Plants were kept in a greenhouse (temperature of 30 ± 2 °C during the day, 10 °C at night and a relative humidity of 70 ± 5%) for two months before starting the experiments. Plants were watered as needed.

Inoculation procedure

The isolate CEBS15 of *C. fimbriata*, used to inoculate the plants, was obtained from symptomatic mango plants collected in Brejo Santo, Ceará State, Brazil. The isolate was preserved using Castellani's method (Dhingra and Sinclair 1995). Plugs of a malt extract agar medium containing fungal mycelia were transferred to Petri dishes containing potato dextrose agar (PDA). After three days, the PDA plugs containing fungal mycelia were transferred to Petri dishes containing the same culture medium and maintained in an incubator (temperature of 25 °C and 12-h photoperiod) for 14 days.

Inoculation was performed following the methods of Araujo et al. (2014a,b). Bark disks (10 mm diameter and 2 mm height) were removed from the stems of plants from both cultivars using a punch. The bark disks were removed approximately 5 cm above the graft scar. A plug (10 mm diameter), removed from the middle portion of each PDA plate obtained from 14-day-old colonies of *C. fimbriata*, was placed in the wound. Each wound containing the fungal mycelia PDA plug was carefully covered with a piece of moistened cotton and enclosed with parafilm to maintain adequate moisture allowing fungal infection. Wounds that only received plugs of PDA medium served as the controls.

Disease assessment

Disease progress in the stem tissue was evaluated at 7, 14 and 21 days after inoculation (dai). For each measured time point, five plants per treatment were eliminated or killed for the measurement of internal necrotic tissue in the scions from cultivars. The upward, downward and radial colonization of the stem tissue by hyphae of *C. fimbriata* were accomplished by measuring the length (in cm) of internal necrotic tissue using an electronic digital caliper (Neiko 01407A, Stainless Steel, Mandaluyong, Philippines) according to Araujo et al. (2014a,b). Symptoms of internal necrotic tissue in both longitudinal and transverse stem sections from scions cultivars caused by *C. fimbriata* infection were photographed using a stereomicroscope (Stemi 2000-C, Carl Zeiss, Germany) coupled to a digital camera (PowerShot A640, Canon).

Processing of stem tissue for analysis of secondary metabolites

Longitudinal stem sections from scions (10 cm length) with wound point centered within the sample were obtained in five plants from Palmer and Ubá cultivars at 7, 14 and 21 dai. Sections taken from the stems from scions of non-inoculated plants served as the controls. The stem sections of all treatments were carefully covered with aluminum foil, placed into plastic bags, frozen in liquid nitrogen and stored at −80 °C (up to one month) until the extraction of the alkaloids and phenolic compounds, as described by Hukkanen et al. (2007), Petkovsek et al. (2009) and Wallis and Chen (2012).

Chemicals

Methanol and the standards caffeic acid, catechin, chlorogenic acid, epicatechin, epigallocatechin, gallic acid, kaempferol, myricetin, *p*-coumaric acid, *p*-hydroxybenzoic acid, phloridizin, protocatechuic acid, salicylhydroxamic acid, sinapinic acid, syringic acid, theobromine, 3-methylxanthine and 7-methylxanthine (Sigma-Aldrich, São Paulo, SP, Brazil) were used to quantify individual alkaloids and phenolics. Purified water was generated from a Millipore Direct-Q® ultrapure water system (Billerica, MA, USA). Standards were dissolved in methanol:water solution (80:20, v/v) at a concentration of 1.0 mg·mL^{-1} and stored at −20 °C for 24 h until the injection in the equipment of ultrapure water.

Extraction of secondary metabolites

Stem sections from scions cultivars were lyophilized using a freeze dryer (maintained at −62 ± 2 °C) model L101 (LIOTOP, Liobras, São Carlos, SP, Brazil) for 72 h and grounded in a ball mill (TECNAL TE 350, Piracicaba,

SP, Brazil) for 4 min. The extraction of soluble secondary metabolites from the stem sections was performed as described by Lisec et al. (2006) and Tohge and Fernie (2010), with some modifications. The fine powder (50 mg) of each lyophilized material was transferred to microcentrifuge tubes and extracted with 1.8 mL of a methanol:water solution (80:20, v/v). The suspension was incubated for 30 min at 30 °C under constant shaking (950 rpm, Thermomixer comfort, Eppendorf, Hamburg, Germany). The extracts from the stem tissue were centrifuged at 11,000 g for 10 min at 4 °C and the supernatant was transferred to new microcentrifuge tubes and stored at −20 °C (up to one month) until the determination of alkaloids and soluble phenolic compounds.

Determination and quantification of secondary metabolites

Soluble compounds present in the supernatant of each sample were analyzed using high-performance liquid chromatography (HPLC) with a Thermo Scientific Accela LC System (Accela PDA detector, Accela autosampler and Accela Pump) (Thermo Fisher Scientific, Austin, TX, USA) that was fitted with a C18 reverse-phase column (RP-18 LiChrospher, 5 μm, 150 × 4.6 mm, Supelco, Bellefonte, PA, USA). The elution solvents were: A (aqueous 0.01 M phosphoric acid) and B (100% methanol). The gradient operation was as follows: 85(A) − 15(B)% over 2 min, 75(A) − 25(B)% over 3 min, 70(A) − 30(B)% over 5 min, 65(A) − 35(B)% over 5 min, 50(A) − 50(B)% over 5 min, 40(A) − 60(B)% over 10 min, 20(A) − 80(B)% over 5 min, 00(A) − 100(B)% over 5 min, 95(A) − 5(B)% over 10 min, 85(A) − 15(B)% over 15 min and 100(A) − 00(B)% over 5 min. A total of 5 μL of each extract was injected into the column, which had a flow rate of 0.5 mL·min⁻¹. The compounds eluted were monitored at 280 nm and were identified by comparing the retention times and spectra as well as by Co-chromatography. The concentration of each alkaloid and soluble phenolic compound was calculated from the peak areas of the sample of the corresponding calibration standard. The threshold for quantification by peak areas was ≈ 10 AU·min⁻¹; therefore, compounds whose peak areas were below this value were not quantified. The concentration of each alkaloid and soluble phenolic compound was expressed as μg·100 mg⁻¹ of dry weight (DW) of stem tissue.

In vitro tests of alkaloids and phenolic compounds against Ceratocystis fimbriata

Standards of alkaloids and phenolics previously identified and quantified in the stem tissue of mango plants were used in vitro against C. fimbriata growth according to the methodology of Rodrigues et al. (2004). The alkaloids (theobromine and 7-methylxanthine) and phenolic compounds (caffeic acid, p-coumaric acid, gallic acid, protocatechuic acid, catechin and epicatechin) were dissolved in methanol:water solution (80:20, v/v) at the concentrations of 10, 20 and 30 mg·mL⁻¹ according to Adrian et al. (1997), Funnell-Harris et al. (2014) and Mace et al. (1985). Petri dishes (9 cm diameter) were filled with 20 mL of PDA and a hole (7 mm diameter) was made in the center of each dish (Rodrigues et al. 2004). A volume of 150 μL of each secondary metabolite solution was transferred to each hole and allowed to dry for 30 min (Rodrigues et al. 2004). In separate Petri dishes, 150 μL of methanol was added to each hole and served as the control (Rodrigues et al. 2004). Two PDA plugs (7 mm diameter), obtained from the margin of an actively growing colony of C. fimbriata, were placed approximately 1.5 cm from the center of each hole (Rodrigues et al. 2004). Plates were transferred to an incubator (25 °C; 12 h light/12 h dark) and the fungal colony diameter was measured once at the same time of the day on multiple days (every 24 h) according to Blodgett and Stanosz (1997) and Funnell-Harris et al. (2014). Radial fungal growth rate (mm/day) was obtained by measuring fungal growth in each dish using an electronic digital caliper and was stopped when two fungal colonies reached confluence in the control treatment (Rodrigues et al. 2004). Representative plates of each treatment were digitally photographed (Coolpix L110; Nikon, Tokyo, Japan) at ten days of incubation to record the pattern of C. fimbriata radial growth.

Experimental design

A 2 × 2 × 3 factorial experiment, consisting of two mango cultivars (Palmer and Ubá), non-inoculated or inoculated plants and three evaluation times (7, 14 and 21 dai), was arranged in a completely randomized design with five replications. Each replication consisted of a plastic plot containing one mango plant. The experiment was repeated once. The in vitro tests were performed twice using four Petri dishes per each concentration (10, 20 and 30 mg·mL⁻¹) of →

the secondary metabolites (theobromine, 7-methylxanthine, caffeic acid, *p*-coumaric acid, gallic acid, protocatechuic acid, catechin and epicatechin) standards. Values were transformed to square root of × (value to be transformed) before statistical analysis. The data were analyzed by analysis of variance (ANOVA) and the treatment means were compared by Tukey's test (p ≤ 0.05) using SAS (Release 8.02 Level 02M0 for Windows, SAS Institute, Inc. 1989, Cary, NC, USA).

RESULTS
Disease assessments

Small internal necrosis at the wound point without any progress in the subsequent evaluation times was observed in the stem tissue of non-inoculated plants from cultivars Palmer (Figure 1a, a1) and Ubá (Figure 1b, b1). The responses of plants from the Palmer and Ubá cultivars to *C. fimbriata* infection were classified as susceptible (Figure 1c, c1, e, e1, g, g1) or resistant (Figure 1d, d1, f, f1, h, h1) reactions, respectively.

Quantification of secondary metabolites

The factor cultivars, plant inoculation and evaluation times as well as their interactions were significant (p ≤ 0.05) for the concentrations of theobromine, 7-methylxanthine, caffeic acid, *p*-coumaric acid, gallic acid, protocatechuic acid, catechin and epicatechin (Figures 2, 3, 4, 5). Both non-inoculated and inoculated plants showed greater concentrations of *p*-coumaric acid in the stem tissue when compared with other secondary metabolites (Figures 2, 3, 4, 5).

Alkaloids

The concentration of theobromine in the stem tissue of non-inoculated plants from cv. Ubá showed a 0.25 – 0.96 fold increase in comparison with the non-inoculated plants from cv. Palmer at 7 and 21 dai (Figure 2a). In contrast, at 14 dai, the concentration of theobromine in the stem tissue of non-inoculated plants from cv. Palmer increased 1.48 fold in comparison with the non-inoculated plants from cv. Ubá (Figure 2a). The concentration of 7-methylxanthine in the stem tissue of non-inoculated plants from cv. Ubá was 0.35 higher than those in the non-inoculated plants from cv. Palmer at 21 dai (Figure 2c). In opposite, at 14 dai, stem tissue of non-inoculated plants from cv. Palmer contained 0.18 more
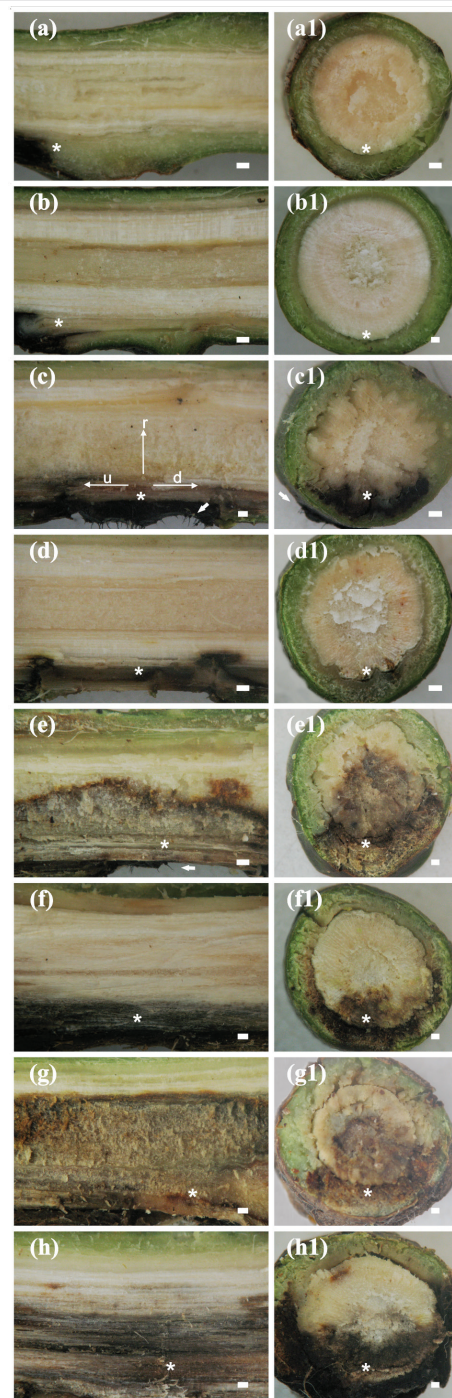
*Stem region where the wound was made and from which the upward (u), downward (d) and radial (r) *C. fimbriata* colonization was measured as detailed in C. The formation of perithecia of *C. fimbriata* was observed at the inoculation point (arrow in c and e). Scale bars: 1.000 μm.

Figure 1. Stem sections of non-inoculated plants of Palmer (a, a1) and Ubá (b, b1) cultivars. Internal necrotic tissue caused by *Ceratocystis fimbriata* infection in longitudinal (c, d, e, f, g, h) and transverse (c1, d1, e1, f1, g1, h1) stem sections of plants from the susceptible cultivar Palmer (a, a1, c, c1, e, e1, g, g1) and the resistant cultivar Ubá (b, b1, d, d1, f, f1, h, h1) at 7 (c, c1, d, d1), 14 (e, e1, f, f1) and 21 (g, g1, h, h1) days after inoculation.

7-methylxanthine in comparison with the non-inoculated plants from cv. Ubá (Figure 2c).

Stem tissue of inoculated plants from cv. Ubá contained 0.33 – 1.37 times more theobromine in comparison with the inoculated plants from cv. Palmer between 7 to 21 dai (Figure 2b). The concentration of 7-methylxanthine in the stem tissue of inoculated plants from cv. Ubá showed a 0.22 – 0.78 fold increase in comparison with the inoculated plants from cv. Palmer from 7 to 21 dai (Figure 2d).

The concentration of theobromine was higher in the stem tissue of non-inoculated plants from cv. Palmer than in the stem tissue of the inoculated ones (Figure 2a, b). In opposite, the concentration of theobromine was higher in the stem tissue of inoculated plants from cv. Ubá than in their counterparts (Figure 2a, b). The concentration of 7-methylxanthine was higher in the stem tissue of non-inoculated plants from both cultivars than in the stem tissue of the inoculated ones (Figure 2c, d).

The concentrations of theobromine and 7-methylxanthine peaked at 14 dai in the stem tissue of non-inoculated plants from Palmer (Figure 2a, c). The concentrations of theobromine and 7-methylxanthine peaked at 7 and 21 dai, respectively, in the stem tissue of non-inoculated plants from cv. Ubá (Figure 2a, c). In the stem tissue of inoculated plants from both cultivars, peaks in the concentrations of
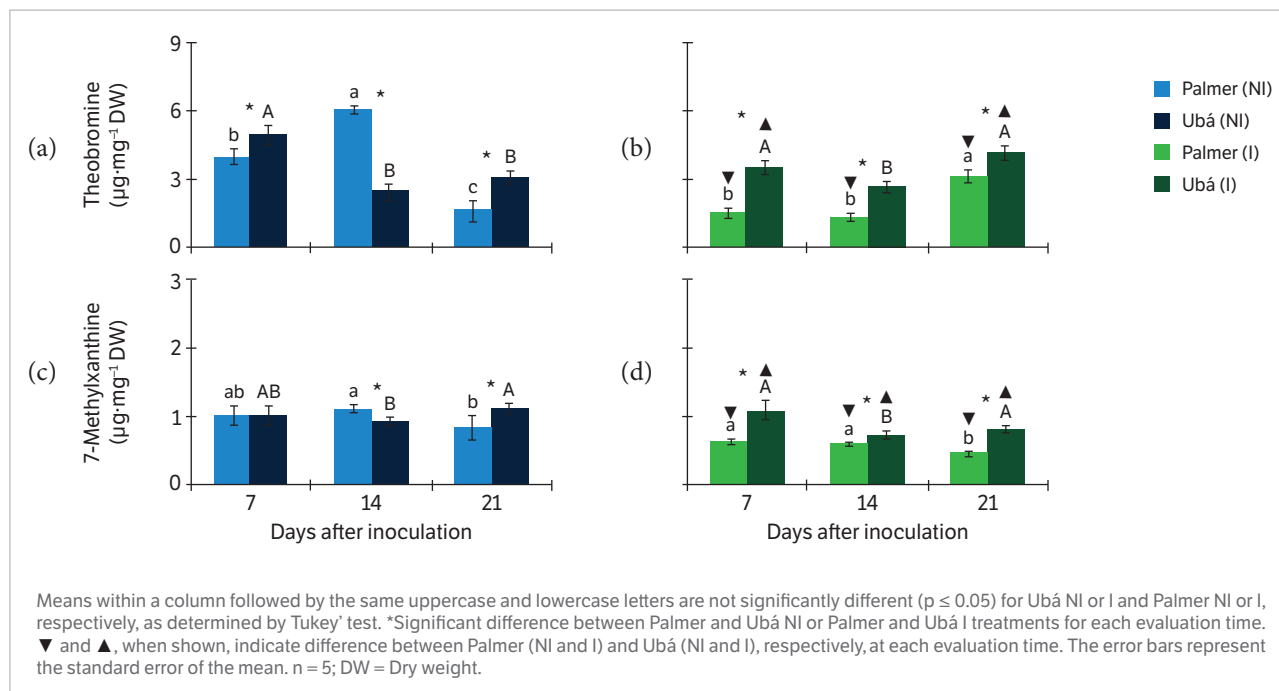
7-methylxanthine and theobromine occurred at 7 and 21 dai, respectively (Figure 2b, d).
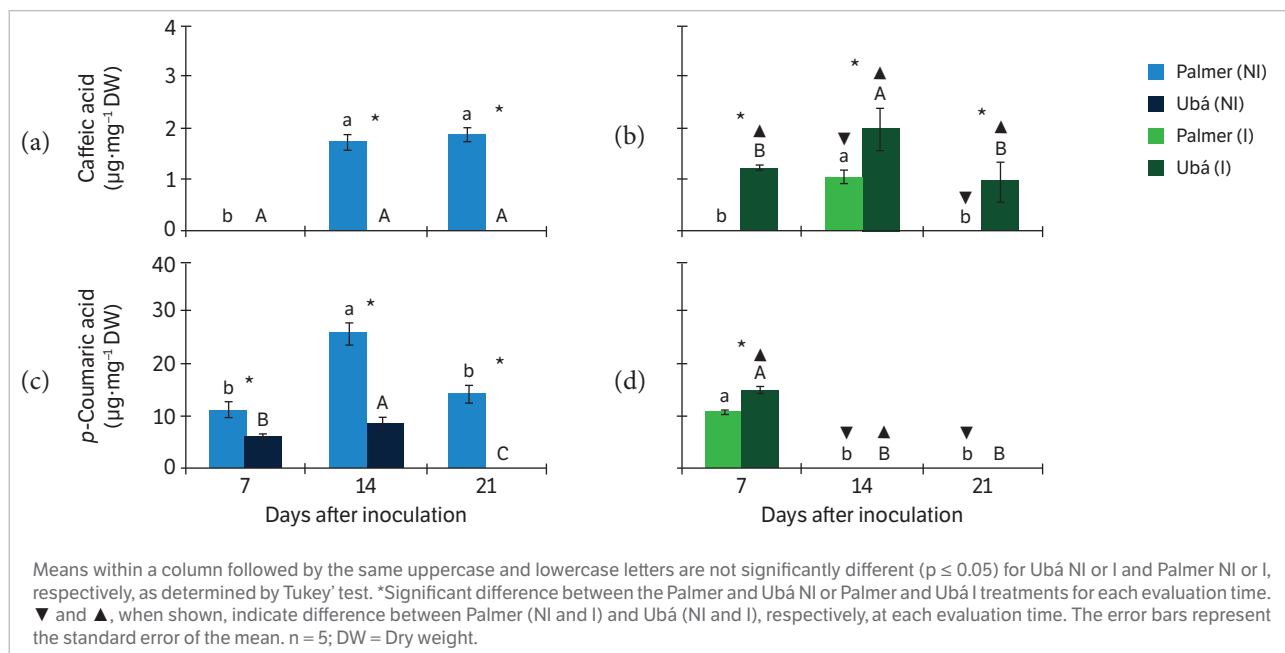
Hydroxycinnamic acids

Caffeic acid was not detected in the stem tissue of non-inoculated plants from cv. Ubá, whereas in the tissue from cv. Palmer the compound was present at 14 and 21 dai (Figure 3a). The concentration of *p*-coumaric acid in the stem tissue of non-inoculated plants from cv. Palmer showed a 0.84 – 1.93 fold increase in comparison with the non-inoculated plants from cv. Ubá at 7 and 14 dai (Figure 3c).

Stem tissue of inoculated plants from cv. Ubá contained 0.90 times more caffeic acid in comparison with the inoculated plants from cv. Palmer at 14 dai (Figure 3b). The concentration of *p*-coumaric acid in the stem tissue of inoculated plants from cv. Ubá showed a 0.40 fold increase in comparison with the inoculated plants from cv. Palmer at 7 dai (Figure 3d).

The concentrations of caffeic acid and *p*-coumaric acid were higher in the stem tissue of non-inoculated plants from cv. Palmer than in the stem tissue of the inoculated ones (Figure 3a, b, c, d). In contrast, the concentrations of caffeic acid and *p*-coumaric acid were higher in the stem tissue of inoculated plants from cv. Ubá than in their counterparts (Figure 3a, b, c, d).

Means within a column followed by the same uppercase and lowercase letters are not significantly different (p ≤ 0.05) for Ubá NI or I and Palmer NI or I, respectively, as determined by Tukey' test. *Significant difference between Palmer and Ubá NI or Palmer and Ubá I treatments for each evaluation time. ▼ and ▲, when shown, indicate difference between Palmer (NI and I) and Ubá (NI and I), respectively, at each evaluation time. The error bars represent the standard error of the mean. n = 5; DW = Dry weight.

Figure 2. Concentrations of the alkaloids — theobromine (a, b) and 7-methylxanthine (c, d) — in the stem tissue of mango plants from Palmer and Ubá cultivars that were not inoculated (NI; a, c) or inoculated (I; b, d) with *Ceratocystis fimbriata* at three different evaluation times.

Means within a column followed by the same uppercase and lowercase letters are not significantly different (p ≤ 0.05) for Ubá NI or I and Palmer NI or I, respectively, as determined by Tukey' test. *Significant difference between the Palmer and Ubá NI or Palmer and Ubá I treatments for each evaluation time. ▼ and ▲, when shown, indicate difference between Palmer (NI and I) and Ubá (NI and I), respectively, at each evaluation time. The error bars represent the standard error of the mean. n = 5; DW = Dry weight.

Figure 3. Concentrations of the hydroxycinnamic acids — caffeic acid (a, b) and p-coumaric acid (c, d) — in the stem tissue of mango plants from Palmer and Ubá cultivars that were not inoculated (NI; a, c) or inoculated (I; b, d) with *Ceratocystis fimbriata* at three different evaluation times.

In the stem tissue of non-inoculated plants from both cultivars, peaks in the concentrations of caffeic acid and p-coumaric acid occurred at 14 dai (Figure 3a, c). The concentrations of p-coumaric acid and caffeic acid peaked at 7 and 14 dai, respectively, in the stem tissue of inoculated plants from both cultivars (Figure 3b, d).

Hydroxybenzoic acids

The concentration of gallic acid in the stem tissue of non-inoculated plants from cv. Ubá showed a 0.67 – 0.70 fold increase in comparison with the non-inoculated plants from cv. Palmer at 7 and 21 dai (Figure 4a). In opposite, the concentration of protocatechuic acid in the stem tissue of non-inoculated plants from cv. Palmer showed a 0.49 – 0.88 fold increase in comparison with the non-inoculated plants from cv. Ubá at 7 and 14 dai (Figure 4c).

Stem tissue of inoculated plants from cv. Ubá contained 0.35 – 0.67 times more gallic acid in comparison with the inoculated plants from cv. Palmer at 7 and 14 dai (Figure 4b). The concentration of protocatechuic acid in the stem tissue of inoculated plants from cv. Ubá increased 0.20 – 0.60 fold in comparison with the inoculated plants from cv. Palmer from 7 to 21 dai (Figure 4d).

The concentration of gallic acid was higher in the stem tissue of non-inoculated plants from both cultivars than in

the stem tissue of inoculated plants (Figure 4a, b), whereas protocatechuic acid only showed greater accumulation in the stem tissue of non-inoculated plants from cv. Palmer (Figure 4c, d). The concentration of protocatechuic acid was higher in the stem tissue of inoculated plants from cv. Ubá than in their counterparts (Figure 4c, d).
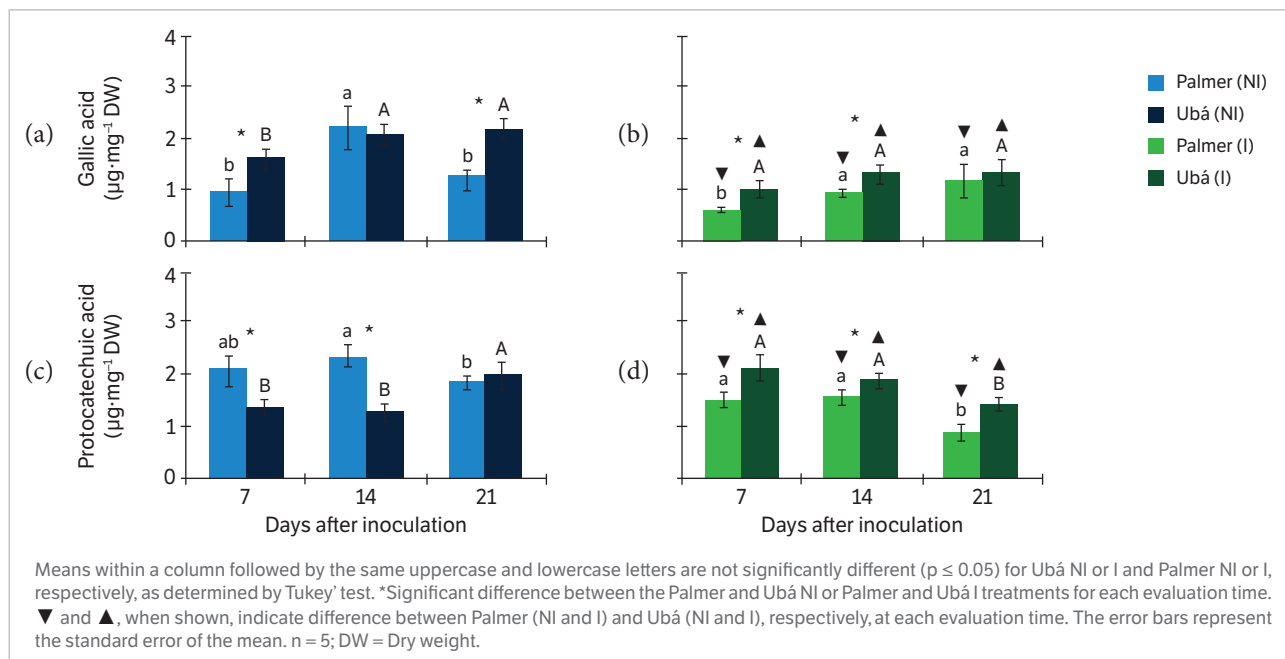
In the stem tissue of non-inoculated and inoculated plants from both cultivars, peaks in the concentration of gallic acid occurred at 14 dai (Figure 4a, c). The concentration of protocatechuic acid peaked at 14 and 21 dai in the stem tissue of non-inoculated plants from cv. Palmer and Ubá, respectively (Figure 4c). In the stem tissue of inoculated plants from both cultivars, peaks in the concentration of protocatechuic acid occurred at 7 dai (Figure 4d).
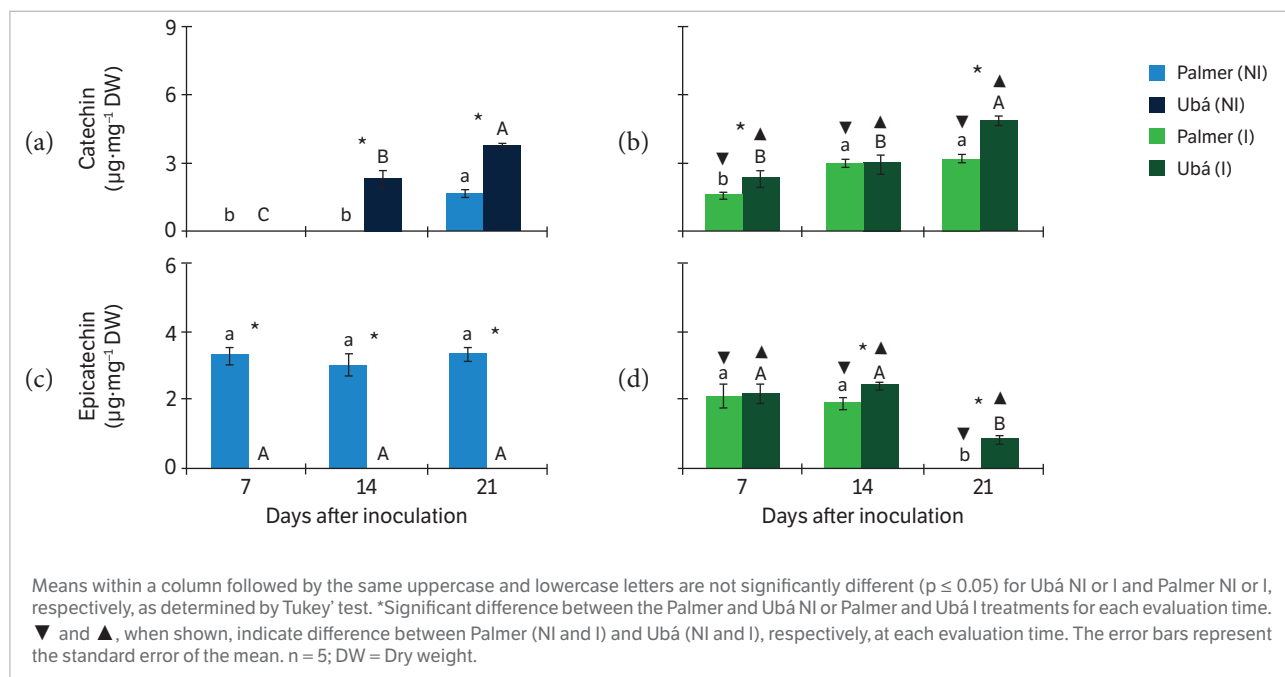
Flavonoids

The concentration of catechin in the stem tissue of non-inoculated plants from cv. Ubá showed a 1.29 fold increase in comparison with the non-inoculated plants from cv. Palmer at 21 dai (Figure 5a). Epicatechin was not detected in the stem tissue of non-inoculated plants from cv. Ubá, whereas, in the tissue from cv. Palmer, the compound was present at all evaluation time (Figure 5c).

Stem tissue of inoculated plants from cv. Ubá contained 0.46 – 0.51 times more catechin in comparison with the

Figure 4. Concentrations of the hydroxybenzoic acids — gallic acid (a, b) and protocatechuic acid (c, d) — in the stem tissue of mango plants from Palmer and Ubá cultivars that were not inoculated (NI; a, c) or inoculated (I; b, d) with *Ceratocystis fimbriata* at three different evaluation times.

Figure 5. Concentrations of the flavonoids — catechin (a, b) and epicatechin (c, d) — in the stem tissue of mango plants from Palmer and Ubá cultivars that were not inoculated (NI; a, c) or inoculated (I; b, d) with *Ceratocystis fimbriata* at three different evaluation times.

inoculated plants from cv. Palmer at 7 and 21 dai (Figure 5b). The concentration of epicatechin in the stem tissue of inoculated plants from cv. Ubá increased 0.27 fold in comparison with the inoculated plants from cv. Palmer at 14 dai (Figure 5d).

The concentration of catechin was higher in the stem tissue of inoculated plants from both cultivars than in the stem tissue of non-inoculated plants (Figure 5a, b). The concentrations of epicatechin were higher in the stem tissue of non-inoculated

plants from cv. Palmer than in the stem tissue of the inoculated ones (Figure 5c, d). In opposite, the concentration of epicatechin was higher in the stem tissue of inoculated plants from cv. Ubá than in their counterparts (Figure 5c, d).

The concentration of catechin peaked at 21 dai in the stem tissue of non-inoculated plants from both cultivars (Figure 5a). Peaks in the concentration of epicatechin were not detected in the stem tissue of non-inoculated plants from both cultivars (Figure 5c). In the stem tissue of inoculated plants from both cultivars, peaks in the concentrations of epicatechin and catechin occurred at 7 and 21 dai, repectively (Figure 5b, d).

In vitro tests

The confluence of *C. fimbriata* colonies for the control treatment occurred at approximately ten days of incubation (Figure 6a,b,c). At the same evaluation time, *C. fimbriata* colonies confluence was not observed on the Petri dishes containing the different concentrations of two alkaloids (theobromine and 7-methylxanthine) (Figure 6a1, b1, c1, a2,b2,c2), two hydroxycinnamic acids (caffeic acid and *p*-coumaric acid) (Figure 6a3, b3, c3, a4, b4, c4), two hydroxybenzoic acids (gallic acid and protocatechuic acid) (Figure 6a5, b5, c5, a6, b6, c6) and two flavonoids (catechin and epicatechin) (Figure 6a7, b7, c7, a8, b8, c8). All secondary metabolites greatly affected fungal growth over time compared to the control treatment (Figure 6). The *C. fimbriata* growth was lower at the highest concentrations (10 to 30 mg·mL^{-1}) mainly for caffeic acid (Figure 6a3, b3, c3), *p*-coumaric acid (Figure 6a4, b4, c4), gallic acid (Figure 6a5, b5, c5) and protocatechuic acid (Figure 6a6, b6, c6) in comparison with the control treatment. The greatest zone of fungal growth inhibition and lowest *C. fimbriata* growth rate was observed on the Petri dishes containing caffeic acid (Figure 6a3, b3, c3) and *p*-coumaric acid (Figure 6a4, b4, c4) treatments.

DISCUSSION

The results from the present study support previous findings that phenolic-like compounds and possibly other antimicrobial compounds belonging to other biochemical classes are involved in mango resistance against *C. fimbriata* infection (Araujo et al. 2014a,b; 2015). To the best of the author's knowledge, however, the present study is the first to describe

the identity of these phenolic and antimicrobial compounds in mango plants with constitutive resistance for mango wilt.
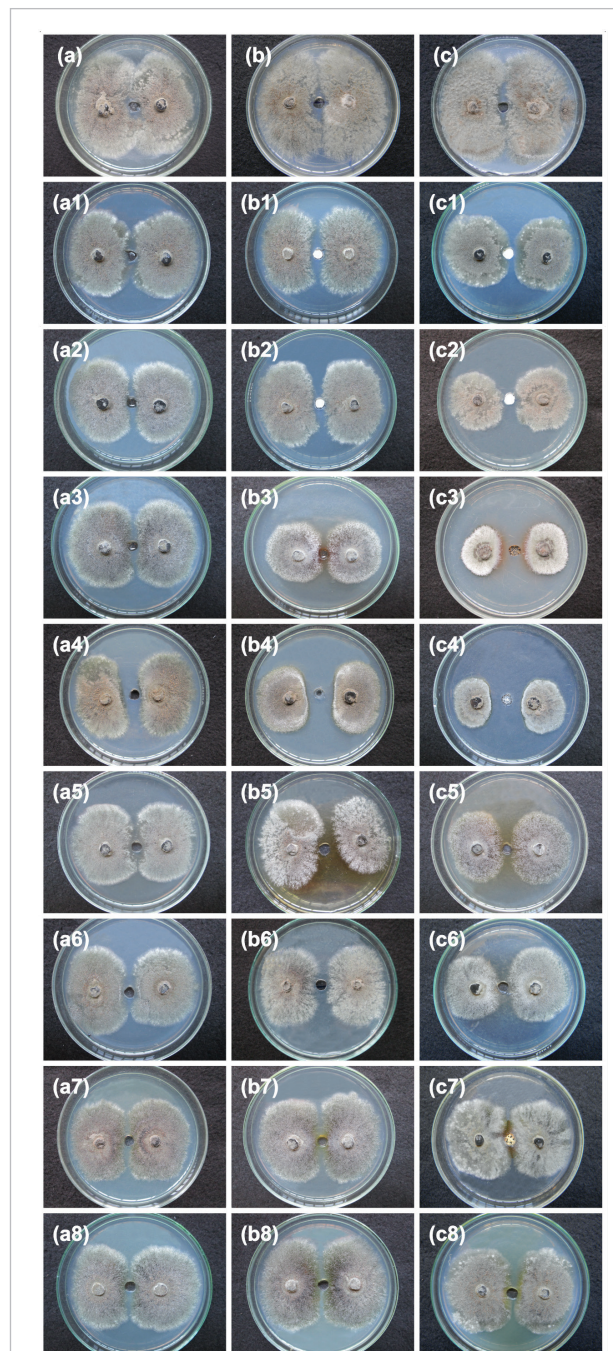
Effect of the concentrations of 10 (a1 to a8), 20 (b1 to b8) and 30 (c1 to c8) mg·mL^{-1} of alkaloids (a1, b1, c1 = theobromine; a2, b2, c2 = 7-methylxanthine), hydroxycinnamic acids (a3, b3, c3 = caffeic acid; a4, b4, c4 = *p*-coumaric acid), hydroxybenzoic acids (a5, b5, c5 = gallic acid; a6, b6, c6 = protocatechuic acid) and flavonoids (a7, b7, c7 = catechin; a8, b8, c8 = epicatechin) on *C. fimbriata* growth in Petri dishes.

Figure 6. *In vitro* tests with alkaloids and phenolic compounds against *Ceratocystis fimbriata*. Confluence of *C. fimbriata* colonies for the control treatment (PDA medium amended with methanol) at ten days of incubation (a, b, c).

The concentration of two alkaloids (theobromine and 7-methylxanthine) and six phenolic compounds (caffeic acid, *p*-coumaric acid, gallic acid, protocatechuic acid, catechin and epicatechin) in the stem tissue of inoculated plants from cv. Ubá was higher in comparison with inoculated plants from cv. Palmer. It has been proven that the concentration of secondary metabolites present in tissue infected by pathogens is cultivar-dependent (Hall et al. 2011; Nicholson and Hammerschmidt 1992; Petkovsek et al. 2009; Prusky and Keen 1993; Treutter 2005; Veberic et al. 2005; Williams et al. 2002). According to Petkovsek et al. (2009) and Veberic et al. (2005), the difference between resistant and susceptible apple cultivars to scab was due to higher concentrations of caffeic acid, catechin, epicatechin, *p*-coumaric acid and protocatechuic acid in the resistant cultivar. High concentrations of methylxanthines (caffeine) and theobromine in the tissue of tobacco and cocoa plants were linked to the induction of plant defense mechanisms against infection by *Moniliophthora perniciosa*, *Pseudomonas syringae* and *Tobacco mosaic virus* (Aneja and Gianfagna 2001; Kim and Sano 2008; Scarpari et al. 2005). It is known that the fungitoxic effect of most plant secondary metabolites on fungal cells is attributed to their interaction with lipids or an increase in phospholipid concentration and therefore membrane permeability, leakage of cellular contents and cytoplasm aggregation (Weete 1980). Araujo et al. (2014a,b) observed an abundance of dead *C. fimbriata* hyphae surrounded by phenolic-like compounds in the cells of stem tissue of plants from cultivars resistant to mango wilt. These findings agree with the data from the present study, which indicated that the concentrations of secondary metabolites were higher in the stem tissue of plants from the resistant cv. Ubá, therefore contributing to a reduction in the symptoms caused by *C. fimbriata* infection. In contrast, in the stem tissue of plants from the susceptible cv. Palmer, the concentration was not sufficient to counteract fungal colonization.

Interestingly, the concentration of most secondary metabolites was higher in the stem tissue of non-inoculated plants from cv. Palmer than in the stem tissue of inoculated ones, while the inverse was observed for plants of cv. Ubá. Plants respond to physical injury, pathogen infection or different types of abiotic stress by increasing the concentration of pre-existing alkaloids and phenolics or by producing new ones through different metabolic pathways (Nicholson and Hammerschmidt 1992; Treutter 2005). Thus, high concentrations of secondary metabolites are often found in the tissue of plants infected by pathogens in comparison with non-infected plants (Aneja and Gianfagna 2001; Hukkanen et al. 2007; Petkovsek et al. 2009; Rodrigues et al. 2004; Scarpari et al. 2005). However, grapevine plants exhibiting symptoms of Pierce's disease caused by *Xylella fastidiosa* (vascular pathogen) showed a significant reduction in phenolic concentrations in the vascular vessels in comparison with non-infected plants (Wallis and Chen 2012). Pierce's disease causes grapevines to enter into a state of unreturned decline; therefore, the plants no longer have the resources to support the production of secondary metabolites with antimicrobial activity (Wallis and Chen 2012). It is known that, in a state of decline, the physiology of the diseased plants cannot be returned to adequate levels, therefore causing them to enter into a survival mode (Park et al. 2013; Wallis and Chen 2012). Thus, higher concentrations of phenolics could be found in tissues of non-infected plants than in the infected ones (Wallis and Chen 2012). In the present study, the intense internal necrotic tissue on the stem of plants from cv. Palmer (due to *C. fimbriata* infection) may have caused the plants to enter into a state of decline that negatively affected the production of secondary metabolites. Alternatively, plants from the resistant cv. Ubá that showed reduced symptoms of mango wilt most likely maintained active defense responses during infection by *C. fimbriata*. Araujo et al. (2015) also reported that plants with induced resistance to mango wilt showed higher concentrations of alkaloids and phenolics during the infection process of *C. fimbrita* in the stem tissue.

Peaks in the concentrations of secondary metabolites in the stem tissue of inoculated plants from both cultivars occurred at early stages of fungal infection. Secondary metabolites are stored in the vacuoles of plant cells from where they can be rapidly infused after wounding, pathogen infection or other types of abiotic stress (Nicholson and Hammerschmidt 1992; Treutter 2005). Higher concentrations of secondary metabolites were detected in the tissue of grapevine, cotton, cacao and mango plants at early stages of *X. fastidiosa*, *F. oxysporum* f. sp. *vasinfectum*, *M. perniciosa* and *C. fimbriata* infections, respectively, but decreased as the disease caused by these pathogens progressed (Hall et al.

2011; Scarpari et al. 2005; Wallis and Chen 2012; Araujo et al. 2015). As a rule, the most resistant plants from a certain cultivar rapidly initiate the production of secondary metabolites in response to pathogens infection (Nicholson and Hammerschmidt 1992; Treutter 2005), explaining, at least in part, the final amount of disease (Wallis and Chen 2012). Araujo et al. (2014a, b) observed that the variation in the susceptibility of mango cultivars to *C. fimbriata* infection was attributed to differences in their ability to reduce fungal spread in the stem tissue through rapid deposition of phenolic-like compounds. In the present study, the concentrations of secondary metabolites in the stem tissue of mango plants were high in the early stages of fungal infection; however, the concentrations in the stem tissue were cultivar-dependent. This could explain why plants from the Palmer cultivar exhibited more rapid and greater disease symptoms than plants from cv. Ubá.

The different concentrations of secondary metabolites added to the Petri dishes inhibited *C. fimbriata* growth over time. According to Funnell-Harris et al. (2014), one possible effect of changes in phenolics composition in the plant is due to their direct effect on inhibiting pathogens growth. The authors provided *in vitro* evidence that ferulic acid inhibited several fungi, which could explain the increase of sorghum lines resistance to some diseases (Funnell-Harris et al. 2014). Blodgett and Stanosz (1997) observed that

different concentrations of phenolic compounds and monoterpenes of red pine inhibited *in vitro* the spore germination and mycelial growth of *Sphaeropsis sapinea*. Blodgett and Stanosz (1997) reported that the changes in the concentration of secondary metabolites may prevent colonization of *S. sapinea* on red pine, explaining, in part, the differential responses of resistant plants to isolates of the fungus. In the present study, it was observed reduced *C. fimbriata* growth at higher concentrations of secondary metabolites during the *in vitro* tests, especially using the caffeic acid and *p*-coumaric acid (hydroxycinnamic acids). Inoculated plants from Ubá cultivar showed the highest concentration of hydroxycinnamic acids in stem tissue, explaining, therefore, why these plants were more resistant to *C. fimbriata* infection than plants from Palmer cultivar.

CONCLUSION

The data from the present study seem to support the concept that secondary metabolites, such as theobromine, 7-methylxanthine, caffeic acid, *p*-coumaric acid, gallic acid, protocatechuic acid, catechin and epicatechin, played an important role in the resistance of mango plants against *C. fimbriata* infection, especially in plants from cv. Ubá.

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