ULTRASTRUCTURAL STUDY OF CONIDIA OF *Colletotrichum gloeosporioides* AND *Colletotrichum musae* TREATED WITH ESSENTIAL OILS

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SUMMARY

Essential oils have shown to be efficient in the control of plant diseases; however, no reports exist regarding their mode of action on plant pathogens. The aim of this work was to evaluate the effect of essential oils from Cymbopogon martinii, Eugenia caryophyllata, Thymus vulgaris, Cinnamomum *sp. and* Cymbopogon citratus *on conidia of* Colletotrichum gloeosporioides *and* Colletotrichum musae, *etiologic agents of anthracnose of guava and banana, respectively, by means of transmission electron microscopy (TEM).* Conidia suspensions *(1×10^{10} conidia/ml) prepared in sterile distilled water with Tween 20® 1.0% were treated with essential oils at 0.5%, remaining under agitation at 25°C for 24h. Water alone was used as control. After centrifugation the supernatant was discarded and the masses of conidia obtained were fixed for 24h in modified Karnovsky fixative. The suspensions were centrifuged again and after discarding the supernatant, the fixed conidia were embedded in agarose gel and subjected to the protocol of sample preparation for TEM, to be observed with a Zeiss EM 109 microscope. The essential oils showed fungitoxic action directly on the conidia of* C. gloeosporioides *and* C. musae, *causing severe damage by promoting cellular disorganization and degradation that makes germination unviable.*

Introduction

The anthracnose caused in avocado, guava, papaya, mango and passion fruit by *Colletotrichum gloeosporioides* and in banana by *Colletotrichum musae* constitutes a major postharvest problem (Peres *et al*., 2002). The essential oils have shown to by efficient in controlling *C. gloeosporioides* (Lee *et al*., 2007; Barrera-Necha *et al*., 2008; Duamkhanmanee, 2008; Rozwalka *et al*., 2008; Sukatta *et al*., 2008; Anaruma *et al*., 2010) and other species of the genus (Ranasinghe *et al*., 2002; Singh *et al*., 2002; Shahi *et al*., 2003; Arroyo *et al*., 2007; Tzortzakis and Economakis, 2007; Tzortzakis, 2009). However, there are no reports regarding the mode of action of these on plant pathogens (Arroyo *et al*., 2007).

Due to the complexity of essential oils, it is assumed that there are multiple mechanisms of action, not well known, that could result on pathogen inhibition, such as protein denaturation, enzyme inhibition and/or membrane disintegration (Janssen, 1989 *apud* Siani *et al*., 2000).

This study aimed to evaluate the mode of action of the essential oils of *Cymbopogon martinii, Eugenia caryophyllata, Thymus vulgaris, Cinnamomum* sp. and *Cymbopogon citratus* on conidia of *Colletotrichum gloeosporioides* and *Colletotrichum musae*, etiologic agents of the anthracnose in guava and banana, respectively, by means of transmission electron microscopy (TEM).

Materials and Methods

Place

The experiments were performed at the Laboratory of Electron Microscopy and Ultrastructural Analysis, Department of Plant Pathology, Universidade Federal de Lavras, Minas Gerais State, Brazil.

Acquisition and maintenance of Colletotrichum gloeosporioides and C. musae isolates

The strains of *C. gloeosporioides* and *C. musae* were obtained from lesions on ripe fruits of guava (cv. Pedro Sato) and banana (cv. Prata), respectively, purchased in the local market and from local producers. After verifying the pathogenicity, the isolates were maintained in potato dextrose agar (PDA), in growth chambers at 25°C and a 12h photoperiod.

Treatments and sample preparation for TEM

For the evaluation of the mode of action of the essential oils on the ultrastructure of *C. gloeosporioides* and *C. musae*, conidia were subjected to treatments containing the essential oils of *Cinnamomum* sp. (cinnamon), *Cymbopogon citratus* (lemongrass), *Eugenia caryophyllata* (Indian clove), *Cymbopogon martinii* (palmarosa), and *Thymus vulgaris* (thyme), selected in function of the potential for total inhibition (100%) on the germination of the pathogens at concentrations of 0.1 and

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ESTUDIO ULTRAESTRUCTURAL DE LAS CONIDIAS DE *Colletotrichum gloeosporioides* Y *Colletotrichum musae* TRATADAS CON ACEITES ESENCIALES

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RESUMEN

Los aceites esenciales han demostrado eficacia en el control de enfermedades de las plantas; sin embargo, no existen reportes del modo de acción de estos sobre los fitopatógenos. El objetivo de este trabajo fue evaluar el efecto de los aceites esenciales de Cymbopogon martinii, Eugenia caryophyllata, Thymus vulgaris, Cinnamomum *sp. y* Cymbopogon citratus *sobre los conidios de* Colletotrichum gloeosporioides *y* Colletotrichum musae, *agentes etiológicos de la antracnosis de la guayaba y del plátano, respectivamente, por medio de microscopía electrónica de transmisión (MET). Una suspensión de conidias (1×10^{10} conidias/ml) preparada con agua destilada estéril y con Tween 20® 1,0%, fue tratada con aceites esenciales al 0,5%, manteniéndose en agitación a 25°C durante 24h. El control consistió de agua solamente. Después de la centrifugación y el descarte del sobrenadante, las masas de conidias obtenidas fueron fijadas por 24h en fijador de Karnovsky modificado. Las suspensiones se centrifugaron de nuevo y después de descartar el sobrenadante, las conidias fijadas fueron incluidas en gel de agarosa y sometidas al protocolo de preparación de muestras para MET. Utilizando un microscopio Zeiss EM 109 se observó que los aceites esenciales ejercieron una acción fungitóxica directa sobre las conidias de* C. gloeosporioides *y* C. musae, *causándoles daños notorios, promoviendo la desorganización y degradación celular que imposibilita la germinación.*

ESTUDO ULTRAESTRUTURAL DE CONÍDIOS DE *Colletotrichum gloeosporioides* E *Colletotrichum musae* TRATADOS COM ÓLEOS ESSENCIAIS

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RESUMO

Os óleos essenciais têm demonstrado eficiência no controle de doenças de plantas; entretanto, praticamente inexistem relatos do modo de ação desses sobre fitopatógenos. O objetivo do trabalho foi avaliar o efeito de óleos essenciais de Cymbopogon martinii, Eugenia caryophyllata, Thymus vulgaris, Cinnamomum *sp. e* Cymbopogon citratus *sobre conídios de* Colletotrichum gloeosporioides *e* Colletotrichum musae, *agentes etiológicos da anthracnose em goiabas e bananas, respectivamente, por meio de microscopia eletrônica de transmissão (MET). Conídios, em suspensão ($1x10^{10}$ conídios.ml) preparada em água destilada e esterilizada com Tween 20® a 1,0%, foram tratados com os óleos essenciais a 0,5% permanecendo sob agitação a 25ºC, por 24 horas. No controle utilizou-se apenas água. Após centrifugação e descarte do sobrenadante, as massas de conídios obtidas foram fixadas por 24h em fixador de Karnovsky modificado. As suspensões foram novamente centrifugadas e após o descarte do sobrenadante, os conídios fixados foram emblocados em gel de agarose e submetidos ao protocolo de preparo de amostras para MET. Nas imagens obtidas no microscópio Zeiss EM 109, observou-se que os óleos essenciais apresentaram ação fungitóxica notória sobre os conídios de* C. gloeosporioides *e* C. musae, *causando danos severos aos mesmos, promovendo a desorganização e degradação celular que inviabilizaram a germinação.*

0.5% observed in previous experiments (data not shown).

Except for the essential oil of *Cinnamomum* sp., extracted by hydrodistillation in a Clevenger type apparatus, the essential oils were supplied by Chamel Industry and Natural Products Commerce, Paraná State, Brazil in 2008. In test tubes, 0.5ml of suspensions of conidia (1×10^{10} conidia/ml) prepared in sterile distilled water with 1.0% Tween 20®, were mixed with 0.5ml of the 1.0% essential oils solutions to obtain a final concentration of 0.5%. For the control, 0.5ml of spore suspensions were mixed with 0.5ml of sterile distilled water. The tubes remained under agitation in an Orbital Shaker at 100rpm and an average temperature of 25°C. After 24h, the contents were transferred to Eppendorf tubes and centrifuged for 3min at 6000rpm. The supernatant was discarded and the masses of conidia fixed in modified Karnovsky fixative, composed of 2.5%glutaraldehyde and 2.5% formaldehyde in 0.05M sodium cacodylate buffer, pH 7.2, plus 0.1M $CaCl_2$, and kept in the refrigerator for 24h (primary fixation). To form pellets, the masses of conidia fixed in Karnovsky were centrifuged for 3min at 6000rpm and the supernatant discarded.

After discarding the supernatant fixative, a 1.0% agarose gel and the pellets were mixed by means of a toothpick and heated at ~45°C, solidifying instantly. The blocks of agarose, after reduction in size, were washed three times for 10min in sodium 0.05 M cacodylate buffer for post-fixation in 2% OsO_4, in a hood, for 4h. The blocks were then washed three times in distilled water and submitted to contrast *en bloc* in an aqueous solution of 0.5% uranyl acetate overnight in the refrigerator. Afterwards, dehydration of the blocks was achieved in an increasing acetone series of 25, 50, 75, 90 and 100% for 10min each, except the last concentration with 3×10min.

The blocks were subsequently embedded, the acetone being replaced by resin in an increasing gradient, remaining 8h in Spurr resin (30%) and acetone (70%), 8h in Spurr resin (70%) and acetone (30%) and 2×24h in pure Spurr resin, at room temperature. Samples were transferred to silicone molds containing polymerized Spurr resin in half of their volume, covered with pure Spurr resin to fill the molds, and kept at 70ºC for 48h for polymerization. The blocks were trimmed with razor blades to a trapezoidal shape with a cutting surface of appropriate size.

Initially, semithin 0.5µm sections were made on Reichert-Jung (Ultracut E) ultramicrotome with a glass knife, for the localization of fungal structures of interest in a light microscope and for orientation of the ultrathin sections. The semithin sections were collected with a gold ring and placed on glass slides, dried in a metal plate at ~60°C, covered with toluidine blue stain (1g toluidine blue, 1g sodium borate and 100ml of water, filtered through a Millipore 0.2µm), heated on a

metal plate until formation of a golden border, washed with distilled water, dried on a hot plate and visualized by light microscopy.

Ultrathin sections (>100nm) were made with a diamond knife, collected on copper grids (300 mesh) previously coated with formvar film, post-contrasted with aqueous solutions of 2% uranyl acetate and 3% lead citrate for 3min on each, and washed with distilled water. The observation was carried out with a transmission electron microscope Zeiss EM 109 at 80kV. The images were digitally recorded and edited in the Photopaint Software of the Corel Draw 13 package.

Results and Discussion

Figures 1a and 2a illustrate the integrity of the cell wall, the plasmatic membrane and cytoplasmatic contents of conidia of *Colletotrichum gloeosporioides* and *C. musae*, respectively, in control, untreated preparations.

In the conidia treated with the essential oils of *C. martinii* (Figures 1b and 2b), *E. caryophyllata* (1c and 2c), *T. vulgaris* (1d and 2d), *C. citratus* (1e and 2e) and *Cinnamomum* sp. (Figures 1f and 2f) changes were observed in the cell wall and in the plasmatic membrane, as well as vacuolization of the cytoplasm.

The number of studies demonstrating the mode of action of essential oils on plant pathogenic fungi is small; however, the results mentioned below, demonstrating cellular structural changes observed in other pathosystems and essential oils, corroborate those obtained in the present study.

Zambonelli *et al.* (2004) verified that the essential oil of *T. vulgaris* (thyme), containing thymol as its main component, caused an increase in the vacuolization of the cytoplasm and an accumulation of lipid droplets, ripples in the plasmalemma and changes in the mitochondria and endoplasmic reticulum of *Colletotrichum lindemuthianum* and *Pythium ultimum*. Rasooli *et al.* (2006) observed severe hyphae collapsing, plasmatic membrane rupture and destruction of mitochondria in *Aspergillus niger* treated with essential oils of *Thymus eriocalyx* and *T. x-porlock*.

The oils of *T. eriocalyx* and *T. x-porlock* were also found to produce irreversible damage to the walls, membranes and cellular organelles by exposing spores of the pathogen. Arroyo *et al.* (2007) found that the volatile compound (E)-hex-2-enal caused changes in the structures of the cell wall and the plasmatic membrane, with consequent disorganization and destruction of organelles and, eventually, cell death of *Colletotrichum acutatum*, one of the agents that cause anthracnose in strawberries.

The accumulation of electron-dense material observed in conidia of *C. gloeosporioides* (Figure 1c) and *C. musae* (Figure 2c) treated with the essential oil of *E. caryophyllata*, characterized by the formation of an electron dark image corroborates the findings of Bakkali (2008), who mentions that, as lipophilic substances, the essential oils penetrate the cell wall and the plasmatic membrane, disrupting the structure of different layers of polysaccharides, fat acids and phospholipids, making them permeable.

The essential oils of *C. citratus* (Figures 1e and 2e) and *Cinnamomum* sp. (Figures 1f and 2f) promoted the leakage of cytoplasmic contents of some conidia. Piper *et al.* (2001) pointed out that substances found in essential oils affect the integrity of cell membranes making them permeable, causing leakage of cellular content.

It was observed that in treatments with the same essential oils occurred variations in ultrastructure of the pathogen's conidia, such as different intensities of vacuolization and leakage of cytoplasmic contents or not. For Knobloch *et al.* (1988) such variations may occur depending on the solubility of essential oils and interaction with the cytoplasmic membrane, thereby determining the antifungal activity. The thickness of the spores' walls may also interfere with the activity of antifungal compounds, as reported by Svircev *et al.* (2007) who did not find any effect of thymol vapors on the cytoplasm of the thick-walled spores of *Monilinia fructicola* in the postharvest treatment of plum.

From the efficacy of essential oils, demonstrated in the total inhibition of the germination of *C. gloeosporioides* and *C. musae*, it can be inferred that such fungitoxic action occurs on other species of *Colletotrichum*, avoiding the dissemination.

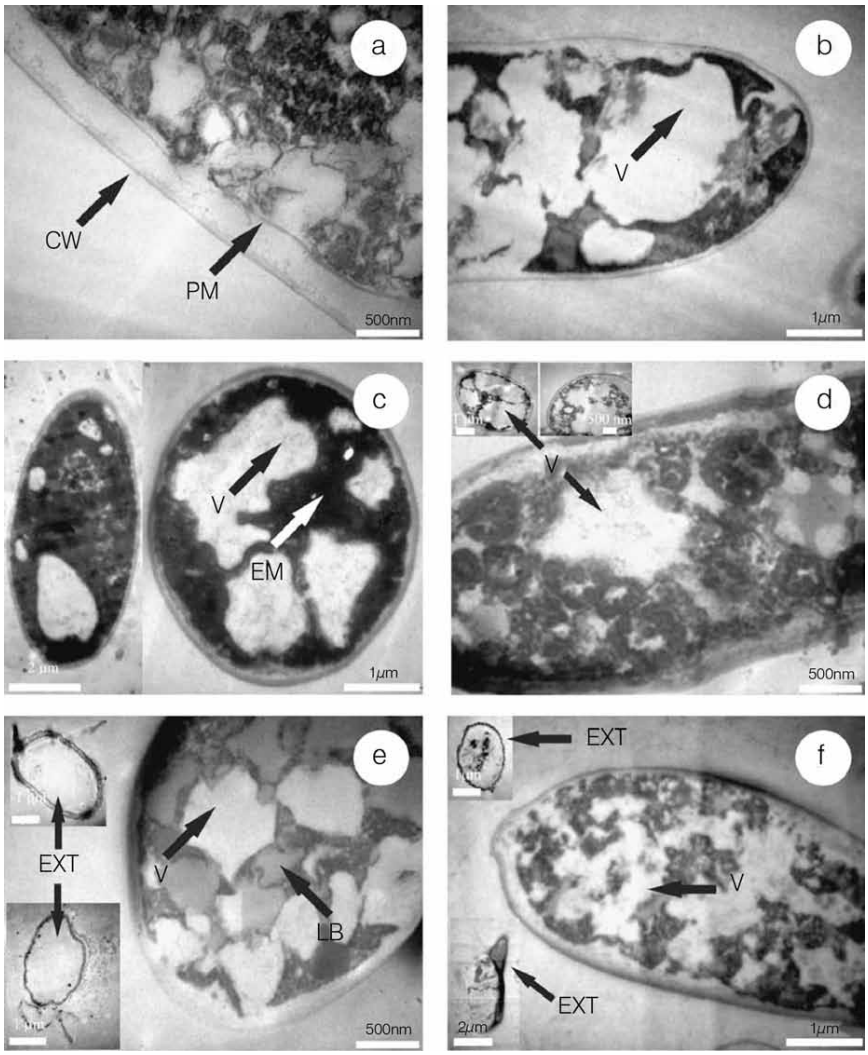

Figure 1. Transmission electron micrographs of untreated conidia of *Colletotrichum gloeosporioides* (control, a), and treated with essential oils of *Cymbopogon martinii* (b); *Eugenia caryophyllata* (c), *Thymus vulgaris* (d), *Cymbopogon citratus* (e) and *Cinnamomum* sp. (f) at 0.5%. CW: cell wall, PM: plasmatic membrane, LB: lipid droplets, V: vacuolization, EM: electron-dense material, and EXT: leakage.

The exploration of antifungal activity of essential oils is presented as an alternative strategy for the control of plant diseases in pre-and post-harvest, representing a lesser risk to human health and environment in pre-and post-harvest and could replace pesticides.

Conclusion

The essential oils of *Cinnamomum* sp., *Cymbopogon citratus*, *Eugenia caryophyllata*, *Cymbopogon martinii* and *Thymus vulgaris* presented direct fungitoxic action on *C. gloeosporioides* and *C. musae*, causing severe damage to cellular ultrastructure of the conidia.

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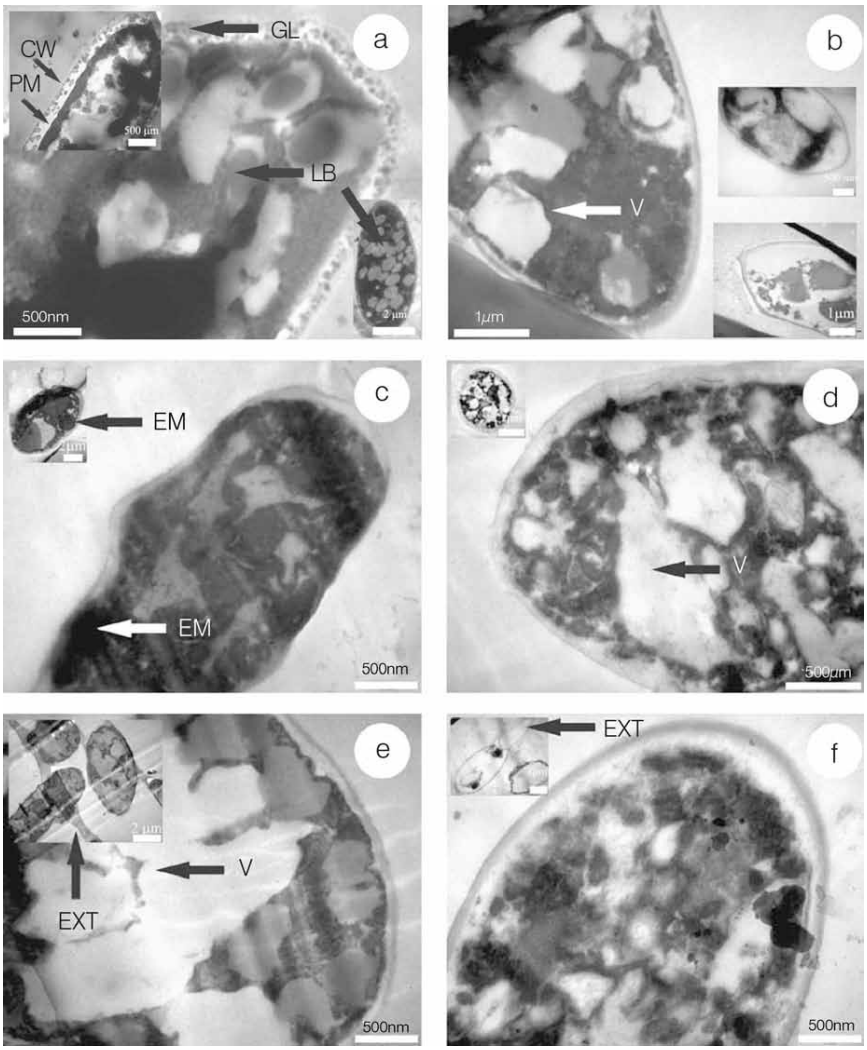

Figure 2. Transmission electron micrographs of untreated conidia of *Colletotrichum musae* (control, a), and treated with essential oils of *Cymbopogon martinii* (b); *Eugenia caryophyllata* (c), *Thymus vulgaris* (d), *Cymbopogon citratus* (e) and *Cinnamomum* sp. (f) at 0.5%. CW: cell wall, PM: plasmatic membrane, LB: lipid droplets, V: vacuolization, EM: electron-dense material, and EXT: leakage.

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