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 martini, 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 be efficient in controlling C. gloeosporioides (Lee et al., 2007; Barre-ra-Necha et al., 2008; Du-amkhannanee, 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; Tzortza-kis, 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 martini, 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).

Material 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 martini (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

KEYWORDS / Alternative Control / Anthracnose / Antifungal Activity / Essential Oils / Transmission Electron Microscopy /

Received: 07/13/2010. Modified: 10/03/2010. Accepted: 10/15/2010.

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912 0378-1844/10/12/912-04 $ 3.00/0 DEC 2010, VOL. 35 Nº 12
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 microscopia electrónica de transmisión (MET). Una suspensión de conidias (1×10^10 conidias/ml) preparada con agua destilada estéril y con Tween 20® a 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 centrífugación y el descarte del sobrenadante, las masas de conidias obtenidas fueron fijadas por 24h en fijador de Karnovsky modificado. Las suspensones 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.

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 CaCl2, 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% OsO4, 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 70°C 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. martini (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 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 pathogens 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 vacuolation of the cytoplasm and an accumulation of lipid droplets, ripples in the plasmalemma and changes in the mitochondria and endoplasmic reticulum of Colletotrichum lindenmuthianum and Pythium ultimum. Rasooli et al. (2006) observed severe hyphae collapsing, plasmatic membrane rupture and destruction of mitochondria in Aspergillus niger treated with the 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 2e) 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.
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 andcould replace pesticides.

**Conclusion**

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

**ACKNOWLEDGMENTS**

The authors acknowledge the doctoral scholarship and financial support granted by the National Council of Scientific and Technological Development (CNPq), and the financial support of the Research Foundation of the State of Minas Gerais (FAPEMIG) to the Laboratory of Electron Microscopy and Ultrastructural Analysis at the Federal University of Lavras, MG, Brazil.

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