CONIDIAL PRODUCTION AND REACTION OF *Alternaria alternata* f. sp. *citri* TO PLANT EXTRACTS

DANIEL DIEGO COSTA CARVALHO

2008

DANIEL DIEGO COSTA CARVALHO

CONIDIAL PRODUCTION AND REACTION OF *Alternaria alternata* f. sp. *citri* TO PLANT EXTRACTS

Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Agronomia, área de concentração Fitopatologia, para a obtenção do título de "Mestre".

Orientador

Prof. Dr. Eduardo Alves

LAVRAS
MINAS GERAIS – BRASIL
2008

Ficha Catalográfica Preparada pela Divisão de Processos Técnicos da Biblioteca Central da UFLA

Carvalho, Daniel Diego Costa.
Conidial production and reaction of *Alternaria alternata* f. sp. *citri* to plant extracts / Daniel Diego Costa Carvalho -- Lavras : UFLA, 2008.
58 p. : il.

Dissertação (Mestrado) – Universidade Federal de Lavras, 2008.
Orientador: Eduardo Alves
Bibliografia.

1. Mancha de Alternaria. 2. Citros. 3. Extratos vegetais. 4. Esporulação. 5. Controle alternativo. I. Universidade Federal de Lavras. II. Título.

CDD – 632.44

DANIEL DIEGO COSTA CARVALHO

CONIDIAL PRODUCTION AND REACTION OF *Alternaria alternata* f. sp. *citri* TO PLANT EXTRACTS

Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Agronomia, área de concentração Fitopatologia, para a obtenção do título de "Mestre".

APROVADA em 15 de fevereiro de 2008

Prof. Dr. José da Cruz Machado	DFP-UFLA
Prof. Dr. Denilson Ferreira Oliveira	DQI-UFLA

Prof. Dr. Eduardo Alves
UFLA
(Orientador)

LAVRAS
MINAS GERAIS – BRASIL
2008

Eu dedico esta dissertação
a quatro pessoas na minha vida:
Helena, minha mãe;
Coriolano, meu pai;
Samara, minha irmã;
e Rafael, meu irmão.

AGRADECIMENTOS

Agradeço a Deus, principal pessoa responsável por esta dissertação de mestrado.

Aos meus pais e meus irmãos, por serem a minha família e por estarem comigo sempre.

À Universidade Federal de Lavras e ao Departamento de Fitopatologia, pela oportunidade de realização do curso de mestrado.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela bolsa de estudos.

Ao Prof. Dr. Eduardo Alves, pela orientação e amizade durante o mestrado.

Aos membros da banca examinadora: Prof. Dr. José da Cruz Machado (DFP) e Prof. Dr. Denilson Ferreira Oliveira (DQI).

Ao Dr. Cristiano Souza Lima (DFP) e ao Prof. Dr. Paulo César Lima (DEX), pela atenção dispensada aos artigos da minha dissertação.

Aos alunos do Laboratório de Produtos Naturais do Departamento de Química da UFLA pela ajuda no preparo dos extratos vegetais.

Aos alunos de iniciação científica Renato Barbosa Camargos e Tereza Raquel Sâmia Batista, pela participação nos experimentos.

Aos laboratoristas do Departamento de Fitopatologia, especialmente Eloísa, Rute, Eliane, Vladimir e Bruno.

Ao amigo Carlos Vinício Vieira.

Aos meus tios e primos, e aos amigos do meu pai em Buriti Alegre - GO.

SUMMARY

PAGE

RESUMO

CARVALHO, Daniel Diego Costa**. Produção de conídios e reação de *Alternaria alternata* f. sp. *citri* a extratos de plantas.** 2008. 58p. (Dissertação - Mestrado em Fitopatologia) – Universidade Federal de Lavras, Lavras, MG[*].

Este trabalho objetivou comparar as metodologias existentes para a produção de conídios de *A. alternata* de dois isolados de tangerina Ponkan, um de limão Cravo, dois de laranja Pêra e um de tangor Murcott e selecionar extratos de plantas potencialmente úteis para o controle da mancha marrom de Alternaria de tangor Murcott, uma doença amplamente disseminada em pomares de citros no Brasil. As metodologias empregadas, para esporulação, foram a produção de conídios, com 12 e 24 horas sob luz branca, avaliação com 24 e 48 horas após estressamento do micélio do fungo, choque térmico, com imediato estressamento do micélio e avaliação com 24 horas, produção de conídios pelo emprego de tecido vegetal sadio e o emprego de lâmpada fluorescente de luz negra NUV. Produção satisfatória de conídios foi obtida com o isolado de *A. alternata* de tangor Murcott, que foi de 2,8 x 10^5 conídios mL^{-1}, mediante emprego da técnica de estressamento da colônia e cultivo do fungo por 24 horas. Os empregos de luz branca (24 h) e negra ultravioleta promoveram expressiva produção de conídios por um isolado de tangerina Ponkan, a qual foi de 17,2 x 10^5 e 10,1 x 10^5 conídios mL^{-1} e por outro de tangor Murcott, a qual foi de 13,9 x 10^5 e 10,1 x 10^5 conídios mL^{-1}, respectivamente. Para alcançar o segundo objetivo deste estudo, 126 extratos de plantas extraídos com metanol (previamente obtidos de várias plantas coletadas no estado de Minas Gerais) foram submetidos a teste *in vitro* em placas de ELISA para verificar atividade sobre a germinação dos conídios. Após a seleção anterior, 20 extratos de plantas foram testados em placas de Petri (6 e 9 cm de diâmetro) para avaliação da germinação dos conídios e crescimento micelial, respectivamente. Além disso, estudos de microscopia eletrônica de varredura mostraram um murchamento dos conídios e uma inibição da emissão do tubo germinativo dos conídios para os extratos de *Ruta graveolens* e *Artemisia annua*, respectivamente. Subsequentemente, 5 extratos de plantas ativos previamente selecionados em condições *in vitro* foram avaliados em frutos de tangor Murcott. Entre os extratos avaliados, o obtido de *Anadenanthera colubrina* (Angico-branco) foi o mais efetivo contra o patógeno, o qual apresentou desempenho similar em relação aos fungicidas avaliados. O extrato causou, aproximadamente, 51% de supressão no desenvolvimento das lesões sobre os frutos, 12 dias após a inoculação, mostrando alto potencial para o controle *in vivo* de *A. alternata*.

---

[*]**Comitê Orientador:** Dr. Eduardo Alves - UFLA (Orientador), Dr. Denilson Ferreira Oliveira – UFLA.

ABSTRACT

CARVALHO, Daniel Diego Costa**. Conidial production and reaction of *Alternaria alternata* f. sp. *citri* to plant extracts.** 2008. 58p. (Dissertation – Plant Pathology) – Federal University of Lavras, Lavras, MG[*].

This study compared existing methodologies to produce conidia of *Alternaria alternata* isolated from Ponkan tangerine (2 isolates), Cravo lemon (1 isolate), Pêra orange (2 isolates) and Murcott tangor (1 isolate) and to select potentially useful plant extracts to the control of Alternaria brown spot (ABS) from Murcott tangor, a widely disseminated disease found in Brazilian citrus orchards. The methodologies used for sporulation were conidia production with 12 and 24 hours under white fluorescent light, evaluation with 24 and 48 hours after applying fungal mycelium stress technique, cold stress followed by injury of mycelium and evaluation with 24 hours, using healthy vegetable tissue and the use of black fluorescent near ultraviolet (NUV) lamp. Satisfactory result was obtained with *A. alternata* isolate from Murcott tangor, with the production of 2.8×10^5 conidia mL^{-1}, when fungal mycelium was stressed (Petri dish with 66.66% of fungi growth) and subsequently 24 h of growth. The use of white light (24 h) and black fluorescent NUV lamp also induced expressive conidia production by one isolate of Ponkan tangerine, which produced 17.2×10^5 and 10.1×10^5 conidia mL^{-1} and another of Murcott tangor, which produced 13.9×10^5 and 10.1×10^5 conidia mL^{-1}, respectively. To the second objective of this study, 126 plant extracts extracted with methanol solvent (previously obtained from several plants collected in Minas Gerais state, Brazil) were tested *in vitro* on ELISA plates for activity on conidia germination. After previous selection, 20 plant extracts were tested on Petri dishes (6 and 9 cm of diameter) for evaluation of conidia germination and mycelium growth, respectively. Besides, scanning electron microscopy study of extract effect on conidia germination showed a conidia dry up and an inhibition of conidia germinative tube by *Ruta graveolens* and *Artemisia annua* extracts, respectively. Subsequently, 5 previously *in vitro* selected active plant extracts were evaluated by assay with Murcott tangor fruits. Among the plant extracts evaluated, the one obtained from *Anadenanthera colubrina* was the more effective against the pathogen, which presented the same performance in relation to the evaluated fungicides. The plant extract was able to cause near to 51% of suppression in the development of ABS on fruits, 12 days after inoculation, showing high potential to *in vivo A. alternata* control.

---

[*]**Guidance Committee**: Dr. Eduardo Alves (Major Professor), Dr. Denilson Ferreira Oliveira.

CHAPTER 1

Comparison of methodologies for pathogenicity and conidial production of *Alternaria alternata* from citrus

Daniel D. C. Carvalho[1]; Eduardo Alves[1*]; Tereza R. S. Batista[2]; Renato B. Camargos[1]; Eloísa A. G. L. Lopes[1].

[1]Departamento de Fitopatologia, Universidade Federal de Lavras, 37200-000, Lavras, MG, Brasil; [2]Centro Universitário de Lavras, 37200-000, Lavras, MG, Brasil.

*Corresponding author. UFLA - Departamento de Fitopatologia, Caixa Postal 3037, cep 37200-000, Lavras, MG, Brasil. Tel.: (55)(35) 3829-1789, Fax: (55)(35) 3829-1795. E-mail: ealves@ufla.br

(Formatted according to *Brazilian Journal of Microbiology)*

Abstract

Conidia production is a problem in the study of *Alternaria alternata* from citrus. Thus, this study was developed to compare existing methodologies to produce conidia of *A. alternata* isolated from Ponkan tangerine (2 isolates), Cravo lemon (1 isolate), Pêra orange (2 isolates) and Murcott tangor (1 isolate). The methodologies used were conidia production with 12 and 24 hours under white fluorescent light, evaluation with 24 and 48 hours after applying fungal mycelium stress technique, cold stress followed by injury of mycelium and evaluation with 24 hours, using healthy vegetable tissue and the use of black fluorescent near ultraviolet (NUV) lamp. Satisfactory result was obtained with *A. alternata* isolate from Murcott tangor, with the production of 2.8 x 10^5 conidia mL^{-1}, when fungal mycelium was stressed (Petri dish with 66.66% of fungi growth) and subsequently 24 h of growth. The use of white light (24 h) and black fluorescent NUV lamp also induced expressive conidia production by one isolate of Ponkan tangerine, which produced 17.2 x 10^5 and 10.1 x 10^5 conidia mL^{-1} and another of Murcott tangor, which produced 13.9 x 10^5 and 10.1 x 10^5 conidia mL^{-1}, respectively. The remaining methodologies analyzed in this study were not able to induce conidia production in satisfactory quantity. The use of both mycelium stress technique and white light (24 h) and black fluorescent NUV lamp allowed the production of enough quantities of conidia to be used *in vitro* (detection of fungitoxic substances) and *in vivo* (pathogenicity test) assays, respectively.

Key words: mycelium stress technique, Murcott tangor, Ponkan tangerine, black fluorescent NUV lamp.

Introduction

Alternaria brown spot (ABS) is an important and serious disease of many tangerines and their hybrids in humid and semiarid areas of citrus cultivation around the world (Timmer et al., 2003). In Brazil, ABS was first found in Rio de Janeiro State (Goes et al., 2001) and subsequently became widespread in the major citrus area in São Paulo State (Peres et al., 2003). During the year of 2006, besides those states, this disease was found in many regions of Minas Gerais State, causing injury to Ponkan tangerine and, especially, to Murcott tangor (Prates, 2007). According to Peres & Timmer (2006), the disease produces black necrotic lesions on young leaves, twigs, and fruit. On leaves, lesions may expand easily due to the production of a host-specific toxin by the pathogen, resulting in leaf drop and twig dieback, in most cases. On fruits, which are very susceptible to ABS, a dry up occurs and lesions vary from small dark necrotic spots to large sunken pockmarks, reducing their value for the fresh fruit market (Vicent et al., 2004).

The causal agent of ABS was originally described as *Alternaria citri* Ellis & Pierce, but further molecular studies showed that all small-spored isolates from citrus were similar. As consequence, they were designated as *A. alternata* f.sp. *citri* (Fr:Fr) Keissl (Peever et al., 2004). Thus, one host-specific pathotype causes disease in tangerines and their hybrids, another pathotype is specific to Rough lemon and Rangpur lime (Peever et al., 2005).

Since conidia production by some *A. alternata* isolates in culture media requires special techniques (Bóveda, 1986; Verzignassi et al., 1997), simple methods to do so are greatly welcome (Balbi-Peña et al., 2006; Peres & Timmer, 2006). Thus, as part of a project aimed to carry out several studies with such fungus, this work was directed towards the establishment of a new methodology to easily obtain *A. alternata* conidia in a culture medium.

Materials and Methods

Fungal isolates

First, fruit peel from symptomatic Murcott tangor, Ponkan tangerine, Pêra orange and Cravo lemon as well as Pêra orange leaves showing symptoms of ABS, were washed in tap water. Then, pieces (10 – 25 mm^2) of these materials were subsequently immersed in 70% ethanol (30-60 s), 2% sodium hypoclorite (30-60 s) and distilled water (2 x 30 s). Four fragments of each material were placed in a Petri dish containing 13 mL of Potato-Dextrose-Agar (PDA) (200 g cooked potato, 20 g dextrose and 20 g agar in 1 L distilled water). After seven days at 25ºC, under photoperiod of 12 h (Prusky et al., 2006), agar plugs (9 mm) with fungal mycelium were transferred to new PDA Petri dishes for fungus purification. The isolates obtained and used in this study are presented in Table 1.

Table 1. Isolates of *Alternaria alternata* from citrus used for the evaluation of conidia production.

Source of isolate	Part of the plant	Isolate code
Cravo lemon	Fruit	B-66-01
Murcott tangor	Fruit	A1-03-04
Pêra orange	Fruit	B-52-09
Pêra orange	Leave	A-07-01
Ponkan tangerine	Fruit	B-52-01
Ponkan tangerine	Fruit	B-62-04

Pathogenicity assay

Ripe and healthy Murcott tangor fruits, Pêra orange, Ponkan tangerine and Cravo lemon were washed with water and soap and left to dry during 60 min. Under aseptic conditions in a laminar flow chamber, four equidistant points

were selected around the point of insertion of the fruit with plant. Three to four perforations (3 mm deep) were made with a needle on each selected point and agar plugs (6 mm) with fungal mycelium of B-66-01, B-52-09, A-07-01 and B-52-01 isolates was placed on each point in the fruit (Dantas et al., 2003). Isolates A1-03-04 and B-62-04, which were able to produce conidia, were inoculated through 20 µL of an aqueous spore suspension at 10^6 conidia mL^{-1} (Colturato, 2006). The inoculated fruits were kept in a moist chamber at 25ºC, under a 12 h photoperiod. After 12 days, symptoms of ABS were verified and the pathogen was re-isolated. The frequency of plant pathogenic fungi was expressed as the percentage of fruits in which the fungus was isolated and the pathogenicity was confirmed (Dantas et al., 2003). Six fruits were used per isolate and the control treatment comprised six fruits in which no fungal inoculation was applied, but only 1% Tween 80.

Conidia production with 12 and 24 hours of dayligth fluorescent lamp

Agar plugs (9 mm) from purified fungus were transferred to PDA plates and after 7 days, under temperature of 25ºC and photoperiod of 12 h (Philips daylight fluorescent lamp, 20W, TLT, 75RS), the conidia production was evaluated (A). In (B) the same methodology was applied, but the photoperiod was 24 h of constant light.

Evaluation with 24 and 48 hours after fungal mycelium stress technique

Agar plugs (9 mm) of purified fungal colonies were transferred to plates and after 7 days, under temperature of 25ºC and photoperiod of 12 h, the mycelium was injuried by the introduction of a needle into the culture, 4 mm deep. Needles were used to make a grid with 7 mm of length inside the blocks up to 66.66% of the fungal growth. The Petri dishes were incubated at

conditions mentioned before and after 24 h (C) and 48 h (D), conidia production was evaluated.

Cold stress followed by injury of mycelium and evaluation after 24 hours

Fungal cultures produced as in the methodology (A), without the application of mycelium stress technique in the same plate, were grown for 7 days, at 66.66% of dish area. After, Petri dishes were transferred to refrigerator, where were kept by 24 h at 5 - 8ºC and without light. Immediately after, the dishes were submitted to mycelium stress technique and transferred to another incubator and after 24 h at 25ºC and photoperiod of 12 h, they were evaluated regarding conidia production by using a Neubauer chamber (E).

Conidia production on citrus plant tissue

Fungal cultures submitted to methodology (A), without mycelium stress technique, were used to originate agar plugs (9 mm) with fungal mycelium. The agar plugs were transferred to new PDA plates and incubated during 5 days, sufficient for the colonization of 50% of the Petri dish area. After that, pieces from fruits and leaves of autoclaved citrus tissue (1 cm^2), of the same species to which the strains was obtained were transferred to PDA plates, next to fungal growth border (F). After 4 days of fungal growth on plant tissues, part of mycelium was removed from the dishes to make microscope mounting using glycerol 50% (v/v) and conidia production was evaluated.

Conidia production by the use of black fluorescent NUV lamp

For the stimuli of conidia production, a methodology developed by Bóveda (1986) was used. It was based on the fungus growth on PDA by 7 days, at 25ºC and photoperiod of 12 h with daylight fluorescent lamp mixed with black fluorescent NUV lamp (Ecolume/NUV, 20W, FL, T-8/BLB) (G). In

addition, the fungal colonies were submitted to mycelium stress technique after 7 days of incubation on black fluorescent NUV lamp, and after 24 h, conidia production was evaluated using a Neubauer chamber (H). The methodology (I) was identical to the (H), excepting incubation time post stress, which was 48 h. A summary of methodologies employed is shown in Table 2.

Table 2. Methodologies employed to conidia production.

Methodology	Summary
A	Conidia production with 12 hours of daylight fluorescent lamp.
B	Conidia production with 24 hours of daylight fluorescent lamp.
C	Evaluation with 24 hours after fungal mycelium stress technique.
D	Evaluation with 48 hours after fungal mycelium stress technique.
E	Cold stress followed by injury of mycelium and evaluation after 24 hours.
F	Conidia production on citrus plant tissue.
G	Use of black fluorescent NUV lamp during 7 days.
H	Use of black fluorescent NUV lamp, followed by mycelium stress and evaluation after 24 hours.
I	Use of black fluorescent NUV lamp, followed by mycelium stress and evaluation after 48 hours.

Evaluation of conidia production on Neubauer chamber

In all methodologies, excepting (F), 10 mL of 1% Tween 80 (g mL^{-1}) autoclaved solution were added and dispersed with glass rod into Petri dishes

followed by filtration. The number of conidia was determined with a Neubauer chamber in a light microscope (Balbi-Peña et al., 2006). All tests were conducted with 4 replicates.

Light microscopy (LM)

Part of mycelium was removed from dishes to make microscope mountings using glycerol 50% (v/v) and the morphology of the fungus was observed using a Leica DME light microscope.

Statistical analysis

Analysis of variance was applied for the data obtained in all methodologies, without any transformation. The Scott-Knott (1974) test ($P \leq 0.05$) was applied.

Results

Pathogenicity assay

Symptoms of Alternaria brown spot were observed in all inoculated fruits (Figure 1), but not on negative control fruits, which were inoculated only with 1% Tween 80. Symptomatic fruits presented *A. alternata* in a 100% frequency.

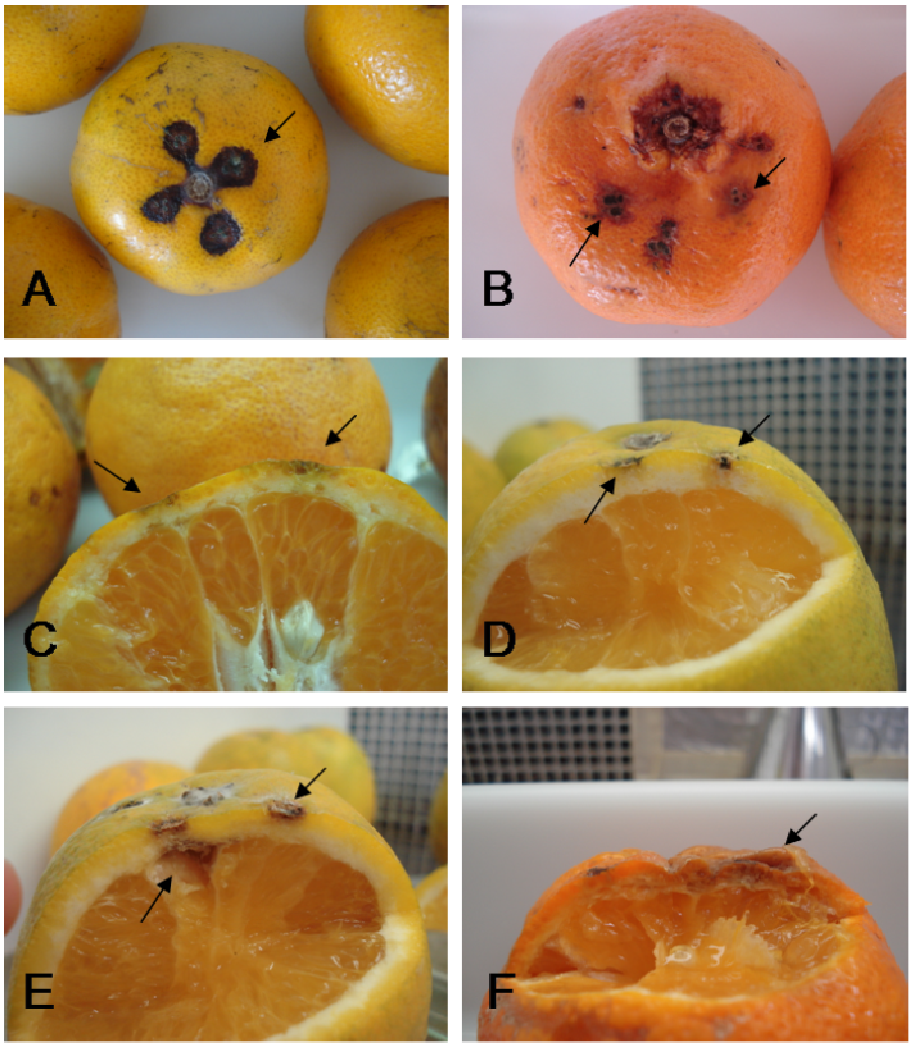

Figure 1. (A - B) Murcott tangor and Ponkan tangerine fruits inoculated with conidia, and (C - F) Ponkan tangerine, Pêra orange and Cravo lemon fruits inoculated with mycelium of *Alternaria alternata,* evaluated 12 days post-inoculation. (A) Murcott tangor (A1-03-04); (B) Ponkan tangerine (B-62-04). (C) Ponkan tangerine (B-52-01); (D) Pêra orange (B-52-09); (E) Pêra orange (A-07-01); (F) Cravo lemon (B-66-01).

Methodologies used for conidia production

Among the 6 isolates studied, only those coming from Murcott tangor (A1-03-04) and Ponkan tangerine fruits (B-62-04) produced considerable amounts of conidia when the mycelium stress technique, fluorescent light white lamp (24 h) and black fluorescent NUV lamp were used (Table 3). Also, some conidia production was obtained with the use of thermal shock. Quantity of conidia considered enough to carry out fungi toxic substances identification was obtained with the isolate obtained from Murcott tangor (A1-03-04) under mycelium stress technique conditions, which produced 2.8 – 3.0 x 10^5 conidia mL^{-1}.

Table 3. Average number of *Alternaria alternata* conidia mL^{-1}* and coefficient of variability (CV) after the application of the methodologies (A), (B), (C), (D), (E), (F), (G), (H) and (I) to the stimulation of *in vitro* conidia production.

Methodology	Isolate						CV
	Cravo lemon fruits	Murcott tangor fruits	Pêra orange fruits	Pêra orange leaves	Ponkan tangerine (B-52-01)	Ponkan tangerine (B-62-04)	
A	-	-	-	-	-	-	-
B	-	13.9 x 10^5 cA	-	-	-	17.2 x 10^5 dA	13.93%
C	-	2.8 x 10^5 aA	-	-	-	4.2 x 10^5 bB	13.40%
D	-	3.0 x 10^5 aA	-	-	-	5.4 x 10^5 bB	8.86%
E	-	1.4 x 10^5 aA	-	-	-	1.4 x 10^5 aA	13.21%
F	-	-	-	-	-	-	-
G	-	10.6 x 10^5 bA	-	-	-	10.1 x 10^5 cA	11.63%
H	-	10.7 x 10^5 bA	-	-	-	10.1 x 10^5 cA	16.52%
I	-	10.0 x 10^5 bA	-	-	-	9.6 x 10^5 cA	4.98%
CV	-	17.61%	-	-	-	12.24%	-

*Means of four replications (4 Petri dishes/treatment, and to each Petri dish, one counting with 8 replications using the Neubauer chamber) with the same small and capital letter in a column and line do not differ significantly ($P \leq 0.05$) according to the Scott-Knott (1974) calculations, respectively.

Characterization of conidia by Light Microscopy (LM)

The length and width of *A. alternata* conidia from Murcott tangor (A1-03-04) and Ponkan tangerine (B-62-04) fruits were measured (Table 4). There were few differences in the size of conidia proceeding from both the host tissue and in the methodology (C).

Table 4. Length and width, beak length, and total length of *Alternaria alternata* conidia from Murcott tangor and Ponkan tangerine (B-62-04) fruits, after the use of methodology (C), in the *in vitro* conidia production.

	Isolated	
	Murcott tangor	Ponkan tangerine
Variable studied*	(A1-03-04)	(B-62-04)
Length (µm)		
Variation	22.5 – 35.0	17.5 – 32.5
Average	28.2	22.8
CV	3.7%	3.0%
Width (µm)		
Variation	5.0 – 10.0	5.0 – 12.5
Average	7.8	8.0
CV	1.6%	1.8%
Beak (µm)		
Variation	2.5 – 7.5	2.5 – 9.5
Average	5.1	4.4
CV	1.7%	2.0%
Total length (µm)		
Variation	22.5 – 40.0	20.0 – 35.0
Average	31.8	25.4
CV	5.1%	4.0%
Conidia with beak	70%	66%

* The key structures of the fungus were measured thirty times; CV: coefficient of variability.

Discussion

The absence of conidia in methodology (A) was not surprising, given that a notable conidia production of *A. alternata* is difficult without interference of physic factors (Bóveda, 1986) or an addition of specific synthetic components at culture medium (Silva & Melo, 1999). Concerning methodology (F), the literature shows that to some fungi, such as *Colletotrichum lindemuthianum* and *Fusarium* spp., the conidia and macroconidia were obtained with insertion of bean pod and carnation leaves into culture medium, respectively (Dalla Pria et al., 2003; Leslie & Summerell, 2006). Similarly, in a specific case, Vakalounakis (1982) obtained a high number of *Alternaria solani* Sorauer conidia through the use of mycelial agar plugs removed from the border of fungal colonies 4 day old and put on Solanaceae leaves. To test this methodology, agar plugs were removed from the border of 4-5 day old colonies, but conidia production was not observed. Also, Timmer et al., (1998) observed that *Alternaria* sp. sporulation was not observed on killed tissues.

The isolates from Cravo lemon (B-66-01), Pêra orange fruits (B-52-09) and leaves (A-07-01) and Ponkan tangerine (B-52-01) did not produce any conidia (Table 3). A possible explanation for these results could be that these isolates are not able to produce conidia in nature (Babu et al., 2004). The virulence level and growth conditions of the isolate can be related with conidia production, because they affect the potential inoculum of *A. alternata* (Masangkay et al., 2000). These authors obtained the most virulent conidia on PDA medium at 28ºC under constant black fluorescent NUV lamp for 4 weeks.

Shahin & Shepard (1979) and Teixeira et al., (2002) observed that black fluorescent NUV lamp is necessary for growth and sporulation of *Alternaria* spp. Furthermore, Ungaro (1981) classified *A. alternata* sporulation as small (less than 10^4 conidia mL^{-1}), medium (10^4 - 10^6 conidia mL^{-1}) and abundant (more than 10^6 conidia mL^{-1}). The methodologies applied to conidia production

to be used in Murcott tangor (A1-03-04) and Ponkan tangerine (B-62-04) isolates, including (C), (D) and (E), can be considered as producing a medium quantity of conidia, except (B), (G), (H) and (I), that produced an abundant quantity (Table 3). Thus, all methodologies that resulted in medium and abundant conidia production were considered to be satisfactory, but (C) and (D) produced the ideal quantity of conidia necessary for *in vitro* assays on ELISA plates, which need 2.6 – 3.0 x 10^5 conidia mL^{-1} (Saks & Barkai-Golan, 1995). On the other hand, according with Colturato (2006), the methodologies (B), (G), (H) and (I) produced a conidia number adequate for *in vivo* tests, which need 10^6 conidia mL^{-1}, such as pathogenicity assay.

It is also worth mentioning that, although without any conidia production, the isolates B-66-01 (Cravo lemon), B-52-09 and A-07-01 (Pêra orange) and B-52-01 (Ponkan tangerine) showed clear symptoms in the pathogenicity assay (Figure 1). A possible explanation for these results could rely on the fact that in the fields, lesions on fruit appear to require more time to produce conidia than in the leaves (Reis & Goes, 2005). Furthermore, Whiteside (1998) noted that, in field, sporulation was more abundant on leaves than on fruit. Even without any conidia production, symptoms on fruit can be verified. Low sporulation was observed by Reis & Goes (2005), 60 days after lesion development, and lesions may be able to produce a few conidia that would serve as primary inoculum in the spring. Also, these authors verified that the sporulation only began about 10 days after symptom development and continued for about the next 40 days, but about 60 days after symptom appearance, conidia were no longer produced (Reis & Goes, 2005).

Surprisingly, the methodology (E) of this work showed that cold stress seems not to be effective in the sporulation. Such behavior is not in accordance with the work of Prasad et al., (1973), wherein *Alternaria solani* showed sporulation under photoperiod of 12 hours and thermal shock at 4ºC. However

the cultures of Prasad were dipped in cold water (4ºC) for 4 min only. In opposition, the objective of thermal shock in this work was to expose the colonies at minimal temperature for longer time and to evaluate the sporulation after subsequently stress of the colonies. Another question rely on temperature, whereas Colturato (2006) revealed that the best temperature for *A. alternata* from Murcott tangor to conidia production was 28ºC, and not 25ºC. Nevertheless high conidia number was obtained at 25ºC in this work (Table 3). This author observed that, at 25ºC, there is a larger mycelial growth. Although Lukens & Horsfall (1973) have reported that the mycelium injury was not benefic to *Alternaria* spp. conidiophore production, this study showed that the introduction of a needle into PDA culture medium at 4 mm deep aiming to cut the fungal mycelium, was advantageous for conidia production. Today there is not enough information about studies for *in vitro* conidia production. Thus, Charlton (1953) observed that *A. solani* fungal colonies, when not injuried, were difficult to remove conidia.

According to the isolate, the methodology applied can be better adjusted to conidia production. An example was the methodologies (C) and (D), which showed higher conidia production to Ponkan tangerine (B-62-04) than Murcott tangor (A1-03-04) (Table 3). This behavior is observed in methodologies that are able to produce lower quantity of conidia.

Regarding length and width of conidia (Table 4), there are not differences among the dimensions of conidia from *A. alternata* coming from different hosts. It is a confirmation that the isolates are from same species, *A. alternata* (Peever et al., 2005). The morphology of conidia, produced under *in vitro* conditions, is in accordance with Ellis (1993), who found out that the length was 20 - 63 µm and the width was 9 - 18 µm for *A. alternata*.

With the improvements of methodologies for conidia production in *A. alternata* obtained in the present study, it is now possible to obtain conidia at

suitable quantity to several purposes, such as to identify fungitoxic substances on ELISA plates and Petri dishes, without using thermic shock, black fluorescent NUV lamp and addition of vegetable tissue on the culture medium to stimulate conidia production. Also, these methodologies can serve as alternatives to induce the sporulation of other *Alternaria* spp. and fungi.

Acknowledgements

The authors thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for a master fellowship and the Doctor Cristiano Souza Lima for revision and suggestion to this paper.

References

BABU, R. M.; SAJEENA, A.; SEETHARAMAN, K. Solid substrate for production of *Alternaria alternata* conidia: a potential mycoherbicide for the control of *Eichhornia crassipes* (water hyacinth). **Weed Research,** Oxford, v. 44, n. 4, p. 298-304, Aug. 2004.

BALBI-PEÑA, M. I.; BECKER, A.; STANGARLIN, J. R.; FRANZENER, G.; LOPES, M. C.; SCHWAN-ESTRADA, K. R. F. Controle de *Alternaria solani* em tomateiro por extratos de *Curcuma longa* e Curcumina - I. Avaliação *in vitro.* **Fitopatologia Brasileira,** Brasília, v. 31, n. 3, p. 310-314, maio/jun. 2006.

BÓVEDA, R. R. M. **Morfologia, patogenicidade, esporulação e sensibilidade a fungicidas *in vitro* de *Alternaria solani* (Ell. e Mart.) Jones e Grout e *Alternaria alternata* (Fr.) Keissler de solanáceas.** 1986. 106 p. Dissertação (Mestrado em Fitopatologia) – Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba.

CHARLTON, K. M. The sporulation of *Alternaria solani* in culture. **Transactions of the British Mycological Society,** New York, v. 36, p. 349-355, 1953.

COLTURATO, A. B. **Efeito do meio de cultura, temperatura, fotoperíodo e fungicidas no crescimento micelial e no controle de *Alternaria alternata* f. sp. *citri*, causador da mancha marrom do tangor Murcote.** 2006. 53 p. Dissertação (Mestrado em Proteção de Plantas) - Universidade Estadual Paulista. Faculdade de Ciências Agronômicas, Botucatu, SP.

DALLA PRIA, M.; AMORIM, L.; BERGAMIN FILHO, A. Quantificação dos componentes monocíclicos da antracnose do feijoeiro. **Fitopatologia Brasileira**, Brasília, v. 28, n. 4, p. 401-407, jul./ago. 2003.

DANTAS, S. A. F.; OLIVEIRA, S. M. A.; MICHEREFF, S. J.; NASCIMENTO, L. C.; GURGEL, L. M. S.; PESSOA, W. R. L. S. Doenças fúngicas pós-colheita em mamões e laranjas comercializados na Central de Abastecimento do Recife. **Fitopatologia Brasileira**, Brasília, v. 28, n. 5, p. 528-533, set./out. 2003.

ELLIS, M. B. **Dematiaceous hyphomycetes.** Oxon: CAB international, 1993. 608 p.

GOES, A.; MONTES DE OCA, A. G.; REIS, R. F. Ocurrencia de la mancha de Alternaria en mandarina Dancy en el estado de Rio de Janeiro. **Fitopatologia Brasileira**, Brasília, v. 26, p. 386, ago. 2001. (Resumo).

LESLIE, J. F.; SUMMERELL, B. A. **The *Fusarium* laboratory manual**. Ames, Iowa: Blackwell Publishing Asia, 2006. 388 p.

LUKENS, R. J.; HORSFALL, J. G. Processes of sporulation in *Alternaria solani* and their response to inhibitors. **Phytopathology,** Saint Paul**,** v. 63, n. 1, p. 176-182, 1973.

MASANGKAY, R. F.; PAULITZ, T. C.; HALLETT, S. G.; WATSON, A. K. Characterization of sporulation of *Alternaria alternata* f. sp sphenocleae. **Biocontrol Science and Technology**, Hants, v. 10, n. 4, p. 385-397, Aug. 2000.

PEEVER, T. L.; CARPENTER-BOGGS, L.; TIMMER, L. W.; CARRIS, L. M.; BHATIA, A. Citrus black rot is caused by phylogenetically distinct lineages of *Alternaria alternata*. **Phytopathology**, Saint Paul, v. 95, n. 5, p. 512–518, May 2005.

PEEVER, T. L.; SU, G.; CARPENTER-BOGGS, L.; TIMMER, L. W. Molecular systematics of citrus-associated *Alternaria* spp. **Mycologia,** Teheran, v. 96, n. 1, p. 119-134, Jan./Feb. 2004.

PERES, N. A. R.; AGOSTINI, J. P.; TIMMER, L. W. Outbreaks of Alternaria brown spot of citrus in Brazil and Argentina. **Plant Disease**, Saint Paul, v. 87, n. 6, p. 750, June 2003.

PERES, N. A.; TIMMER, L. W. Evaluation of the Alter-Rater model for spray timing for control of Alternaria brown spot on Murcott tangor in Brazil. **Crop Protection**, Oxon, v. 25, n. 5, p. 454–460, May 2006.

PRASAD, B.; DUTT, B. L.; NAGAICH, B. B. Inducing sporulation in *Alternaria solani* I. Effect of water treatment. **Mycopathologia et Mycologia Applicata,** Dordrecht, v. 49, n. 2-3, p. 141-146, 1973.

PRATES, H. S. **Mancha de alternaria das tangerinas**. Campinas, SP: Secretaria de Agricultura e Abastecimento, 2007. 4 p. Disponível em: <http://www.cati.sp.gov.br/novacati/tecnologias/doencas_de_plantas/alternaria/mancha_alternaria.htm>. Acesso em: 27 fev. 2007.

PRUSKY, D.; KOBILER, I.; AKERMAN, M.; MIYARA, I. Effect of acidic solutions and acidic prochloraz on the control of postharvest decay caused by *Alternaria alternata* in mango and persimmon fruit. **Postharvest Biology and Technology**, Amsterdam, v. 42, n. 2, p. 134-141, Nov. 2006.

REIS, R. F.; De GOES, A. Effect of lesion age, humidity, and fungicide application on sporulation of *Alternaria alternata*, the cause of brown spot of tangerine. **Plant Disease**, Saint Paul, v. 90, n. 8, p. 1051-1054, Aug. 2005.

SAKS, Y.; BARKAI-GOLAN, R. Aloe Vera gel activity against plant pathogenic fungi. **Postharvest Biology and Technology**, Amsterdam, v. 6, n. 1-2, p. 159-165, June 1995.

SCOTT, A. J.; KNOTT, M. A. A cluster analyses method for grouping means in the analyses of variance. **Biometrics**, Washington, v. 30, n. 3, p. 507-512, 1974.

SHAHIN, E. A.; SHEPPARD, J. F. An efficient technique for inducing profuse sporulation of *Alternaria* species. **Phytopathology,** Saint Paul**,** v. 69, n. 6, p. 618-620, 1979.

SILVA, C. M. M. S.; MELO, I. S. Requisitos nutricionais para o fungo *Alternaria alternata*. **Pesquisa Agropecuária Brasileira,** Brasília, v. 34, p. 499-503, mar. 1999.

TEIXEIRA, H.; ARIAS, S. M. S.; CHITARRA, L. G.; MACHADO, J. C. Eficiência comparativa de lâmpadas fluorescentes na detecção e quantificação de fungos em sementes. **Ciência e Agrotecnologia**, Lavras, v. 26, n. 2, p. 259-268, mar./abr. 2002.

TIMMER, L. W.; PEEVER, T. L.; SOLEL, Z.; AKIMITSU, K. Alternaria diseases of citrus - novel pathosystems. **Phytopathologia Mediterranea,** Florence, v. 42, n. 2, p. 99–112, Aug. 2003.

TIMMER, L. W.; SOLEL, Z.; GOTTWALD, T. R.; IBANEZ, A. M.; ZITKO, S. E. Environmental factors affecting production, release, and field populations of conidia of *Alternaria alternata*, the cause of brown spot of citrus. **Phytopathology**, Saint Paul, v. 88, n. 11, p. 1218-1223, Nov. 1998.

UNGARO, M. R. G. **Sensibilidade de *Alternaria alternata* (Fr.) Keissler a três fungicidas.** 1981. 67 p. Dissertação (Mestrado em Genética e Melhoramento de Plantas) – Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, SP.

VAKALOUNAKIS, D. J. An efficient and simple technique for inducing profuse sporulation in *Alternaria solani*. **Phytopathologia mediterranea,** Bologna, v. 21, n. 43, p. 89-90, 1982.

VERZIGNASSI, J. R.; VIDA, J. B.; HOMECHIN, M. Ocorrência e transmissão de *Alternaria steviae* e *A. alternata* em sementesde *Stevia rebaudiana* (bert.) Bertoni. **Revista Brasileira de Sementes,** Londrina, v. 19, n. 2, p. 283-287, 1997.

VICENT, A.; BADAL J.; ASENSI, M. J.; SANZ, N.; ARMENGOL, J.; GARC´IA-JIM´ENEZ, J. Laboratory evaluation of citrus cultivars susceptibility and influence of fruit size on Fortune mandarin to infection by *Alternaria alternata* pv. c*itri.* **European Journal of Plant Pathology**, Dordrech, v. 110**,** n. 3, p. 245–251, Mar. 2004.

WHITESIDE, J. O. Alternaria brown spot of mandarins. In: WHITESIDE, J. O.; GARNSEY, S. M.; TIMMER, L. M. **Compendium of citrus diseases**. Saint Paul: American Phytopatological Society, 1988. p. 8.

CHAPTER 2

Activity of plant extracts against *Alternaria alternata* from Murcott tangor

Daniel D. C. Carvalho [1], Eduardo Alves * [1], Renato B. Camargos [1], Denilson F. Oliveira [2], José R. S. Scolforo [3] and Douglas A. de Carvalho [4]

[1]Universidade Federal de Lavras (UFLA), Departamento de Fitopatologia, C.P. 3037, CEP 37.200-000, Lavras (MG), Brazil (Phone: (55)(35)3829-1789; fax: (55)(35)3829-1283; e-mail: ealves@ufla.br); [2]UFLA, Departamento de Química, C.P. 3037, CEP 37.200-000, Lavras (MG), Brazil; [3]UFLA, Departamento de Ciências Florestais, C.P. 3037, CEP 37.200-000, Lavras (MG), Brazil; [4]UFLA, Departamento de Biologia, C.P. 3037, CEP 37.200-000, Lavras (MG), Brazil.

* Author for correspondence

(Formatted according to *European Journal of Plant Pathology)*

Abstract

This work selected potentially useful plant extracts for controlling Alternaria *alternata* from Murcott tangor, a widely disseminated disease found in Brazilian citrus orchards. Initially, an experiment to evaluate the effect on the fungal growth by 126 plant extracts obtained by methanol extraction of several species collected in Minas Gerais State, Brazil, were carried out in 96-well polypropylene plates. Only the extracts from *Artemisia annua*, *Anadenanthera colubrina* and *Ruta graveolens* presented activity. When such extracts were submitted to conidia germination and mycelium growth assays, the best results were obtained with *A. colubrina*, whose extract could act efficiently against conidia and mycelium. Scanning electron microscopy allowed the observation that *A. colubrina* was more efficient than some commercial fungicides to prevent conidia germination. Thus, it is possible to conclude that such plant presents high potential to control *A. alternata* in Murcott tangor.

Key words: Alternaria brown spot, *Artemisia annua, Ruta graveolens, Anadenanthera colubrina*

Introduction

According to Peres & Timmer (2006), the alternaria brown spot (ABS), caused by the fungus *Alternaria alternata* (Fr:Fr) Keissl, produces black necrotic lesions on leaves of tangerines (*Citrus reticulata* Blanco) and their hybrids, which may expand easily due to the production of a host-specific toxin by the pathogen, resulting in leaf drop and twig dieback. On fruits, which are very susceptible to infections according to Vicent et al. (2004), it causes lesions that vary from small dark necrotic spots to large sunken pockmarks. Such a disease has expanded worldwide, becoming a serious problem in humid and semiarid citrus orchards (Peres & Timmer, 2006). In Brazil, ABS was first found in the State of Rio de Janeiro (Goes et al., 2001) and subsequently became widespread in the States of São Paulo and Minas Gerais, the main Brazilian citrus areas (Peres & Timmer, 2006).

The methods to control ABS are based on the use of fungicides, which increase production costs and contaminate foods and the environment with toxic substances (McFayden, 1998). Thus, cheaper and less toxic products active against *A. alternata* are greatly required (Bowers & Locke, 2000; Lindsey & Staden, 2004). Since plants are considered sources of environmental friendly substances to control plant diseases (Thangavelu et al., 2004), in order to contribute for the development of new methods to control *A. alternata* on Murcott tangor (probable *Citrus sinensis* (L.) Osb. x *C. reticulata* Blanco hybrid), several plant extracts were evaluated in relation to their ability to produce active substances against such fungus.

Materials and methods

Preparation of plant extracts

Initially, parts of 105 plants (Table 1) were collected in the Alto Rio Grande and São Francisco River regions of Minas Gerais State, Brazilian southeast region, and identified by comparison with specimens available in the Herbarium ESAL-Universidade Federal de Lavras during the year of 2005. They were dried in an oven at 40^{o}C during 48 h, grounded and exhaustively extracted with methanol at room temperature. The solvent present in the liquid phase of each solution was removed under vacuum to afford 126 extracts that were stored at -10^{o}C until the moment to be used.

Table 1 Plants collected in Alto Rio Grande and São Francisco river regions of Minas Gerais State, Brazil.

Plant extracts	Part of plant	Family
Achillea millefolium L.	Leaves and Flowers	Asteraceae
Ageratum conyzoides L.	Leaves	Asteraceae
Albizia polycephala (Benth.) Killip	Leaves	Fabaceae
Allophylus edulis (A.St.-Hil.) Radlk.	Leaves and Barks	Sapindaceae
Amaioua guianensis Aublet	Barks	Rubiaceae
Anadenanthera colubrina (Vell.) Brenan	Barks	Fabaceae
Annona cacans Warm.	Barks	Annonaceae
Anonna squamosa L.	Leaves	Annoneceae
Artemisia absinthium L.	Leaves	Compositae
Artemisia annua L.	Leaves	Asteraceae

Table 1. Continued...

Artemisia vulgaris L.	Leaves	Asteraceae
Baccharis trimera L.	Leaves	Asteraceae
Bathysa meridionalis Smith & Downs	Barks	Rubiaceae
Brugmansia suaveolens (Willd.) Bercht. & Presl	Barks	Solanaceae
Cabralea canjerana (Vell.) Mart.	Leaves and Barks	Meliaceae
Calendula officinalis L.	Leaves and Flowers	Asteraceae
Callisthene major Mart.	Barks	Vochysiaceae
Calyptranthes clusiifolia (Miq.) O.Berg	Barks and Leaves	Myrtaceae
Cariniana estrellensis (Raddi) Kuntze	Leaves and Barks	Lecythidaceae
Cariniana legalis (Mart.) Kuntze	Barks	Lecythidaceae
Celtis iguanaea (Jacquin) Sargent	Leaves and Barks	Ulmaceae
Centella asiatica (L.) Urban	Leaves	Apiaceae
Chenopodium ambrosioides L.	Leaves	Chenopodiaceae
Citrus aurantium L.	Leaves	Rutaceae
Coffea arabica L.	Leaves	Rubiaceae
Coix lacryma-jobi L.	Leaves	Poaceae
Cordia ecalyculata Vell.	Barks	Boraginaceae
Croton floribundus Sprengel	Barks	Euphorbiaceae
Croton urucurana Baillon	Leaves and Barks	Euphorbiaceae
Cryptocarya aschersoniana Mez	Leaves and Barks	Lauraceae
Cupania vernalis Cambess	Barks	Sapindaceae
Curcuma longa L.	Leaves	Zingiberaceae

Table 1. Continued...

Cynara scolymus L.	Leaves	Asteraceae
Daphnopsis fasciculata (Meisner) Nevling	Barks	Thymelaeaceae
Datura metel L.	Leaves	Solanaceae
Dendropanax cuneatus (DC.) Decne & Planchon	Barks	Araliaceae
Digitalis lanata Ehrh.	Leaves	Scrophulariaceae
Eclipta alba (L.) Hassk	Leaves	Asteraceae
Equisetum arvense L.	Stalks	Equisetaceae
Eugenia florida DC.	Leaves	Myrtaceae
Euphorbia tirucalli L.	Stalks	Euphorbiaceae
Fícus carica L.	Leaves	Moraceae
Fícus trigona L.f.	Barks	Moraceae
Foeniculum vulgare Miller	Leaves and Stalks	Apiaceae
Ginkgo biloba L.	Leaves	Ginkgoaceae
Glechoma hederacea L.	Leaves	Lamiaceae
Guazuma ulmifolia Lam.	Barks	Sterculiaceae
Hedera helix L.	Leaves	Araliaceae
Hypericum perforatum L.	Leaves	Clusiaceae
Ixora warmingii Müll. Arg.	Leaves and Barks	Rubiaceae
Jatropha curcas L.	Leaves and Flowers	Euphorbiaceae
Justicia pectoralis Vault.	Leaves	Acanthaceae
Laurus nobilis L.	Leaves	Lauraceae
Lavandula officinalis Chaich	Leaves	Lamiaceae
Leonurus sibiricus L.	Leaves	Lamiaceae
Malva sylvestris L.	Leaves	Malvaceae

Table 1. Continued...

Mangifera indica L.	Leaves	Anacardiaceae
Melissa officinalis L.	Leaves	Labiatae
Mentha arvensis L.	Leaves	Lamiaceae
Mentha longifolia (L.) Hudson	Leaves	Labiatae
Mentha piperita L.	Leaves	Lamiaceae
Mentha pulegium L.	Leaves	Labiatae
Mentha spicata L.	Leaves	Lamiaceae
Mimosa pudica L.	Leaves and Flowers	Fabaceae
Momordica charantia L.	Leaves	Cucurbitaceae
Musa sapientum L.	Leaves	Musaceae
Nepeta cataria (Catnip.)	Leaves	Lamiaceae
Nicotiana tabacum L.	Leaves	Solanaceae
Ocimum basiculum L.	Leaves	Labiatae
Ocimum gratissimum L.	Leaves	Lamiaceae
Origanum vulgaris L.	Leaves	Labiatae
Petiveria alliacea L.	Leaves	Phytolaccaceae
Piper tuberculatum Jacq.	Leaves	Piperaceae
Plantago lanceolata L.	Leaves	Plantaginaceae
Plantago major L.	Leaves	Plantaginaceae
Porophyllum ruderale (Jack.) Cass.	Leaves	Compositae
Protium heptaphyllum (Aublet) Marchand	Leaves	Burseraceae
Psidium guajava L.	Leaves	Myrtaceae
Pteridium aquilinum L.	Leaves	Polypodiaceae
Punica granatum L.	Leaves	Liapunicaceae
Rhamnidium elaeocarpum Reissek	Barks	Rhamnaceae

Table 1. Continued...

Ricinus communis L.	Leaves	Euphorbiaceae
Rosamarinus officinalis L.	Leaves	Labiatae
Ruta graveolens L.	Flowers and Leaves	Rutaceae
Salvia officinalis L.	Leaves	Lamiaceae
Sambucus nigra L.	Leaves and Flowers	Caprifoliaceae
Schinus terebinthifolius Raddi	Leaves	Anarcadiaceae
Solanum argenteum Dunal	Leaves and Barks	Solanaceae
Sonchus oleraceous L.	Leaves	Asteraceae
Styrax pohlii A.DC.	Leaves	Styracaceae
Symphytum officinale L.	Leaves	Boraginaceae
Tagetes spp. L.	Leaves and Flowers	Asteraceae
Taraxacum officinale Cass.	Leaves	Compositae
Terminalia brasiliensis Camb.	Barks and Leaves	Combretaceae
Tetradenia riparia (Hoechst) NE. Br	Leaves	Lamiaceae
Thymus vulgaris L.	Leaves	Lamiaceae
Tilia cordata Mill	Leaves	Tiliaceae
Tithonia diversifolia (Hemsl.) Gray	Leaves	Asteraceae
Trichilia clausseni C.DC.	Barks	Meliaceae
Trichilia hirta L.	Barks	Meliaceae
Tropaeolum majus L.	Leaves and Flowers	Tropaeoloceae
Urtiga dioica L.	Leaves	Urticaceae
Vochysia tucanorum Mart.	Barks	Vochysiaceae

Table 1. Continued...

Zanthoxylum pohlianum Engl.	Barks	Rutaceae
Zingiber officinale Rosc.	Leaves	Zingiberaceae

Fungus isolation

Pieces (10 – 25 mm^2) of washed fruit barks from Murcott tangor (probable *Citrus sinensis* (L.) Osb. x *C. reticulata* Blanco hybrid) were subsequently immersed into 70% ethanol (30-60 s), 2% sodium hypoclorite (30-60 s) and distilled water (2 x 30 s). Four fragments were placed in a Petri dish containing potato-dextrose-agar (PDA) (200 g cooked potato, 20 g dextrose, 20 g agar and 1 l distilled water). After seven days at 25ºC, under a 12 h photoperiod, 9 mm disks of the medium containing the fungus mycelium were transferred to new Petri dishes containing the same culture medium, to identify *A. alternata* in accordance with Prusky et al. (2006). Briefly, healthy Murcott tangor fruits were washed, dried and inoculated with *A. alternata* (20 µl of an aqueous suspension at 10^6 conidia ml^{-1}) through 3 mm deep perforations (16 per fruit, divided in 4 inoculation points) by the use of a needle. After 12 days in a moist chamber at 25ºC, under a 12 h photoperiod, appearance of ABS symptoms was considered a confirmation of the fungus identity (Dantas et al., 2003; Colturato, 2006) (Figure 1).

Fungus growth assay

Plant extracts (2 mg) were dissolved in 500 µl of an aqueous 1% (g ml^{-1}) Tween 80 solution and mixed with 100 µl of an *A. alternata* conidia suspension at 2.6 – 3.0 x 10^5 conidia ml^{-1}. Into each 300 µl cell of a 96-wells polypropylene plate containing 130 µl of PDA with Terramicin – Oxitetracicline cloridrate 500 mg (Pfizer, 0.55 mg ml^{-1} PDA) were poured 20 µl of the resulting suspension. After three days at 25ºC, under a 12 h photoperiod (Bóveda, 1986), plant

extracts that prevented fungal growth were considered active. This experiment was carried out with four replicates, using an aqueous *A. alternata* conidia suspension at 4.3 - 5.0 x 10^4 conidia ml^{-1} in 1% Tween 80 as negative control and a 3.5 mg ml^{-1} Dacobre PM (chlorotalonil 250 g kg^{-1} and copper oxychloride 504 g kg^{-1}, produced by Iharabras S. A. Chemicals Industries) solution in 1% Tween 80 as a positive control. A 0.16 mg ml^{-1} Amistar (Azoxistrobin 500g kg^{-1}, produced by Syngenta Crop Protection) solution in 1% Tween 80 was also used as positive control.

Conidia germination assay

Plant extracts (4 mg) were dissolved in 1.0 ml of an aqueous 1% (g ml^{-1}) Tween 80 solution and mixed with 100 µl of an *A. alternata* conidia suspension at 2.6 – 3.0 x 10^5 conidia ml^{-1}. 520 µl of the final suspension was added to 4.0 ml of a solidified water-agar (WA) medium (20 g agar, 555 mg Tetracycline and 1 l distilled water) in a 6.0 cm Petri dish. After 12 h at 25°C, under illumination, conidia were considered germinated when their germinative tube lengths were larger or equal to the smaller conidia diameter (Balbi-Peña et al., 2006). This experiment was carried out with four repetitions (50 conidia each), using an aqueous *A. alternata* conidia suspension at 2.1 – 2.5 x 10^4 conidia ml^{-1} in 1% Tween 80 as negative control and 3.5 mg ml^{-1} Dacobre PM and 0.16 mg ml^{-1} Amistar solutions in 1% Tween 80 as positive controls.

Mycelium growth assay

Plant extracts dissolved (7 mg ml^{-1}) in 1% Tween 80 were added to 9 cm Petri dishes (0.5 ml/dish) containing PDA (8 ml/dish) with tetracycline (Bunker, 0.55 mg ml^{-1} PDA). Subsequently, a 9 mm PDA disk with *A. alternata* mycelium, obtained from a seven-day old colony (1.8 cm from the colony center), was placed upside down on the center of each dish. After seven days at

25°C, under a 12 h photoperiod, the colony diameter in each dish was measured and data were converted into percentage. This experiment was done with three repetitions, using 1% Tween 80 as negative control and 3.5 mg ml^{-1} of Dacobre PM and 0.16 mg ml^{-1} of Amistar solutions in 1% Tween 80 as positive controls.

Scanning electron microscopy

Just after the fungus growth assay evaluation, PDA disks (6 mm) from treatments with *A. colubrina*, *R. graveolens*, *A. annua*, Dacobre PM, Amistar and 1% Tween 80 (negative control) were removed from the polypropylene plates and submitted to the procedure described by Alves (2004). Right after this, each disk was fixed in a modified Karnovsky solution (2.5% Glutaraldehyde, 2% Paraformaldehyde in a 0.05 M sodium cacodilate buffer at pH 7.2 containing 0.001 M $CaCl_2$) during 48 h, washed three times with the same buffer for 30 min, post-fixed during 2 h in a 1% osmium tetroxide solution in 0.05 M sodium cacodilate buffer at pH 7.2, washed three times with distilled water, dehydrated in a gradient series of acetone solutions (25, 50, 75, 90 and 100%) and dried with carbon dioxide in a critical point dryer (Bal-tec CPD 030). Then, disks were mounted on aluminum stubs with double-sided tape and coated by vacuum evaporation, with a gold layer of 20 nm (Bal-tec SCD 050). All samples were observed in an Evo40 Leo scanning electron microscope.

Statistical analysis

Data from the conidia and mycelium inhibition assays were submitted to analysis of variance and average values were compared by Scott-Knott (1974) calculations ($P \leq 0.05$). Statistical analyses were done using SISVAR software (Ferreira, 2000).

Results

Fungus isolation

Symptoms of ABS were verified in all inoculated fruits, confirming *A. alternata* as the isolated fungus (Figure 1).

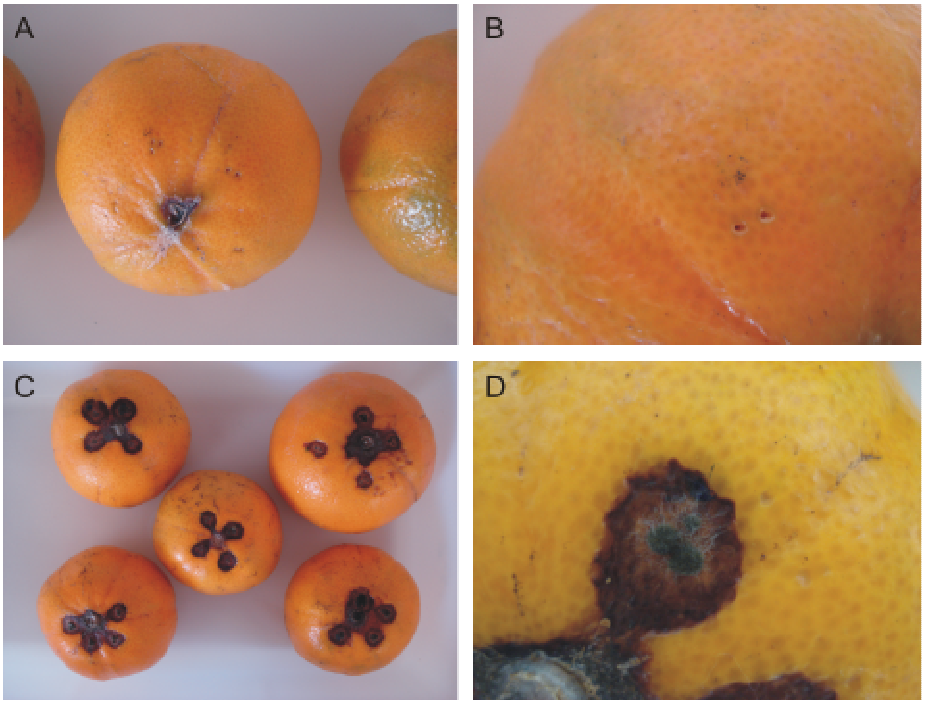

Fig. 1 Pathogenicity assay: (**A**) Fruit inoculated with 1% Tween 80. (**B**) Detail (3 mm deep perforations) of negative control. (**C**) Fruits inoculated with conidia from the isolated fungus, which caused symptoms of Alternaria brown spot. (**D**) Detail of brown spot.

Fungus growth assay

Among the 126 extracts studied, only those from leaves of *Artemisia annua*, barks of *Anadenanthera colubrina* and a mixture of flowers and leaves of *Ruta graveolens*, were active against *A. alternata* (Table 2).

Table 02 Effect of plant extracts on *Alternaria alternata* from Murcott tangor during the fungus growth, conidia germination and mycelium growth assays.

Plant	Fungus growth assay**	Germinated conidia*	Mycelium growth (%)*
Achillea millefolium (leaves)	-	31.5 c	88 c
Achillea millefolium (flowers)	-	36.5 c	95 c
Anadenanthera colubrina (barks)	+	3.0 a	54 a
Artemisia annua (leaves)	+	20.7 b	57 a
Cariniana estrellensis (leaves)	-	45.0 e	91 c
Cariniana estrellensis (barks)	-	33.5 c	66 b
Citrus aurantium (leaves)	-	35.5 c	93 c
Croton urucurana (leaves)	-	34.2 c	74 b
Datura metel (leaves)	-	44.2 e	101 d
Ficus carica (leaves)	-	23.2 b	106 d
Ficus trigona . (barks)	-	41.0 d	78 b
Glechoma hederacea (leaves)	-	46.7 e	93 c
Guazuma ulmifolia (barks)	-	42.2 d	87 c
Jatropha curcas (flowers)	-	46.0 e	125 e
Ocimum basiculum (leaves)	-	41.7 d	108 d
Origanum vulgaris (leaves)	-	40.5 d	104 d
Plantago lanceolata (leaves)	-	34.5 c	105 d
Ruta graveolens (flowers and leaves)	+	34.5 c	72 b
Trichilia clausseni (barks)	-	46.2 e	87 c
Trichilia hirta (barks)	-	39.2 d	85 c
Chlorotalonyl and Copper oxychloride***	+	3.5 a	55 a
Azoxistrobin****	+	7.2 a	52 a
1% Tween 80	-	44.0 e	100 d
Coefficient of variability		11.17%	6.48%

* Means of four replicates with the same letter in a column do not differ significantly ($P \leq 0.05$) according to the Scott-Knott (1974) calculations; ** (+): Absence of mycelium growth, (-): Presence of mycelium growth; *** 0.87 and 1.76 g l^{-1}, respectively; **** 0.08 g l^{-1}.

Conidia and mycelium growth assays

During the conidia germination assay (Table 2), carried out with 20 plant extracts, the best result was obtained with *A. colubrina*. The corresponding extract afforded value statistically identical to those obtained with the commercial fungicides used as positive controls. Although less efficiently, the extracts of *A. annua* and *R. graveolens* also inhibited *A. alternaria* conidia germination. Surprisingly, most of the other extracts also presented weak activity against the fungus. During the mycelium growth assay (Table 2), the extracts of *A. colubrina* and *A. annua* presented the lowest values, which were statistically identical to those obtained with commercial fungicides. Among the other extracts, only five of them presented no activity.

Scanning electron microscopy

No conidia could be detected during the scanning electron microscopy (SEM) of samples exposed to *A. colubrina* extract. Nevertheless, a shrinking of those exposed to *R. graveolens* extract was observed, while conidia submitted to *A. annua* extracts presented shorter germinative tube than those exposed to the negative control (1% Tween 80). It was also possible to detect some hyphae on samples from experiments with *R. graveolens* and *A. annua* extracts, but not with *A. colubrina* (Figures 2 and 3).

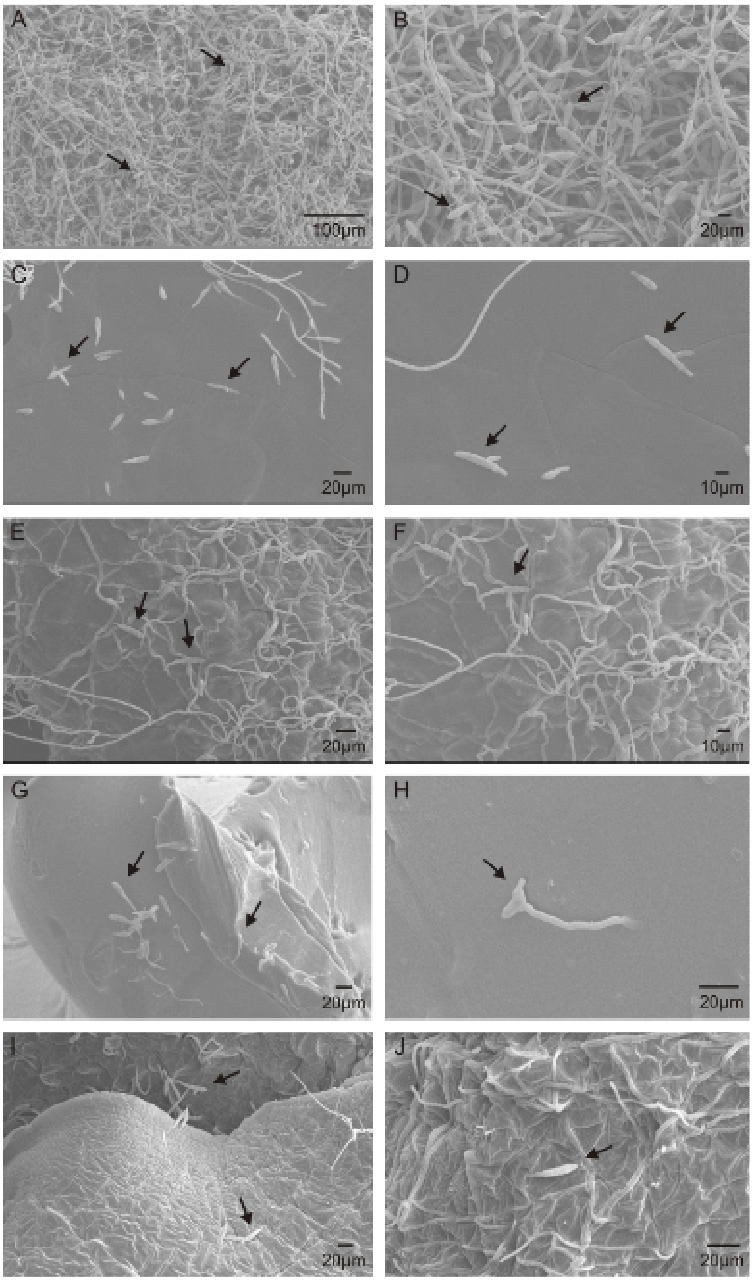

Fig. 2 Scanning electron micrographs of *Alternaria alternata* conidia and mycelium exposed to: (**A, B**) 1% Tween 80. (**C, D**) *Artemisia annua* extract. (**E, F**) *Ruta graveolens* extract. (**G, H**) Dacobre PM. (**I, J**) Amistar.

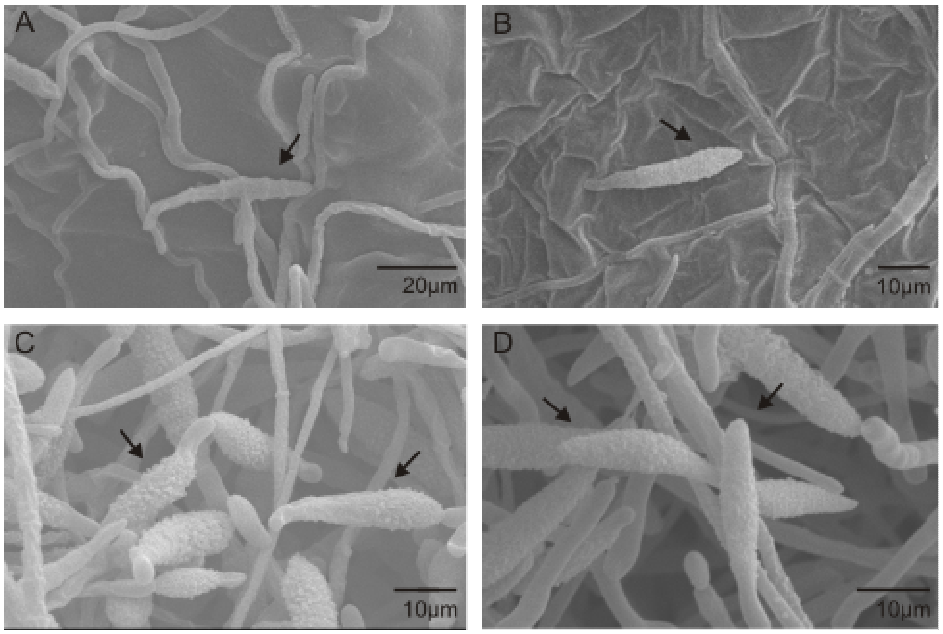

Fig. 3 Scanning electron micrograph details of *Alternaria alternata* conidia exposed to *Ruta graveolens* extract, Amistar fungicide and 1% Tween 80. (**A**) Severe conidia shrinking caused by exposition to *R. graveolens* extract. An arrow show the rings (septa) enhanced by whither of cells. (**B**) Conidia shrinking after treatment with Amistar, but with less intensity than the one observed in *R. graveolens* extract. (**C**, **D**) Conidia after exposition to 1% Tween 80. No deformity is observed.

Discussion

During the first experiment, carried out in 96-well polypropylene plates, only extracts from *A. colubrina*, *A. annua* and *R. graveolens* presented potential to control *A. alternata*. Although the activity of the last two plants against other fungi was already described in the literature (Soylu et al., 2005; Meepagala et al., 2005), no previous report on the antifungal activity of *A. colubrina* could be found.

When submitted to the conidia and mycelium inhibition assays, *A. colubrina* presented values statistically identical to those obtained with the commercial fungicides. As a consequence, it was possible to conclude that such plant presents great potential to be used in *A. alternata* control. A lower efficiency was observed for *A. annua*, whose extract afforded value for germinated conidia statistically higher than those obtained when using commercial fungicides. Among the three active plant extracts in the fungus growth assay, the worse was *R. graveolens*, since its extract lacked in efficiency on both conidia and mycelium inhibition assays.

Other plant extracts were also used during the conidia and mycelium inhibition assays, since a comparison between these experiments and the fungus growth assay was desired. Although most of them presented values statistically different from the negative control (1% Tween 80), none of them was as efficient as *A. colubrina* extract. It is also possible to notice that the extracts of *C. estrellensis* and *C. urucurana* presented values statistically identical to those obtained with *R. graveolens* extract. Thus, it seems that those three plant extracts are very close to the detection limit of the fungus growth assay.

The results discussed so far were confirmed by SEM, since no conidium or hypha could be observed on samples previously treated with *A. colubrina* extracts. Probably, the substances produced by such plant prevented the tube germination emission. As a consequence, conidia could not adhere to the culture

medium and must have been lost during the sample preparation for SEM. Even the commercial fungicides were not so efficient, since it was possible to visualize some germinated conidia and hyphae after the treatment with Dacobre PM and Amistar.

Although *R. graveolens* extract was not as efficient as the one of *A. colubrina*, it caused conidia shrinking (Figures 2 and 3). This effect was similar to the one observed in Amistar (azoxistrobin), while *A. annua* extract presented similar effect to that of Dacobre PM (chlorotalonil and copper oxychloride) (Figure 2). However, inhibition of germinative tube emission was more premature for *A. annua* extract than for Dacobre PM.

Considering the above results, it is possible to conclude that *A. colubrina* presents great potential to control *A. alternata* in Murcott tangor. Thus, future studies to evaluate the extracts of such plant under field conditions should be carried out.

Acknowledgements

The authors gratefully acknowledge CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for a master fellowship and CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for financial support.

References

ALVES, E. **Curso introdutório à microscopia eletrônica de varredura**. Lavras: UFLA/FAEPE, 2004. 43 p.

BALBI-PEÑA, M. I.; BECKER, A.; STANGARLIN, J. R.; FRANZENER, G.; LOPES, M. C.; SCHWAN-ESTRADA, K. R. F. Controle de *Alternaria solani* em tomateiro por extratos de *Curcuma longa* e Curcumina - I. Avaliação *in vitro.* **Fitopatologia Brasileira,** Brasília, v. 31, n. 3, p. 310-314, maio/jun. 2006.

BÓVEDA, R. R. M. **Morfologia, patogenicidade, esporulação e sensibilidade a fungicidas *in vitro* de *Alternaria solani* (Ell. e Mart.) Jones e Grout e *Alternaria alternata* (Fr.) Keissler de solanáceas.** 1986. 106 p. Dissertação (Mestrado em Fitopatologia) – Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, SP.

BOWERS, J. H.; LOCKE, J. C. Effect of botanical extracts on the population density of *Fusarium oxysporum* in soil and control of Fusarium wilt in the greenhouse. **Plant Disease,** Saint Paul, v. 84, n. 3, p. 300-305, Mar. 2000.

COLTURATO, A. B. **Efeito do meio de cultura, temperatura, fotoperíodo e fungicidas no crescimento micelial e no controle de *Alternaria alternata* f. sp. *citri*, causador da mancha marrom do tangor Murcote.** 2006. 53 p. Dissertação (Mestrado em Proteção de Plantas) - Faculdade de Ciências Agronômicas da Universidade Estadual Paulista, Botucatu, SP.

DANTAS, S. A. F.; OLIVEIRA, S. M. A.; MICHEREFF, S. J.; NASCIMENTO, L. C.; GURGEL, L. M. S.; PESSOA, W. R. L. S. Doenças fúngicas pós-colheita em mamões e laranjas comercializados na central de abastecimento do recife. **Fitopatologia Brasileira**, Brasília, v. 28, n. 5, p. 528-533, set./out. 2003.

FERREIRA, D. F. Análises estatísticas por meio do Sisvar para Windows versão 4.0. In: REUNIÃO ANUAL DA REGIÃO BRASILEIRA DA SOCIEDADE INTERNACIONAL DE BIOMETRIA, 45., 2000, São Carlos. **Anais...** São Carlos: Universidade Federal de São Carlos, 2000. p. 255-258.

GOES, A.; MONTES DE OCA, A. G.; REIS, R. F. Ocurrencia de la mancha de Alternaria en mandarina Dancy en el estado de Rio de Janeiro. **Fitopatologia Brasileira**, Brasília, v. 26, p. 386, ago. 2001. Resumo.

LINDSEY, K. L.; STADEN, J. V. Growth inhibition of plant pathogenic fungi by extracts of *Allium sativum* and *Tulbaghia violacea*. **South African Journal of Botany,** Grahamstown, v. 70, n. 4, p. 671-673, Oct. 2004.

McFAYDEN, R. E. C. Biological control of weeds. **Annual Review of Entomology,** Palo Alto, v. 43, p. 369-393, 1998.

MEEPAGALA, K. A.; SCHRADER, K. K.; WEDGE, D. E.; DUKE, S. O. Algicidal and antifungal compounds from the roots of *Ruta graveolens* and synthesis of their analogs. **Phytochemistry**, Oxford, v. 66, n. 22, p. 2689-2695, Nov. 2005.

PERES, N. A.; TIMMER, L. W. Evaluation of the Alter-Rater model for spray timing for control of Alternaria brown spot on Murcott tangor in Brazil. **Crop Protection**, Oxon, v. 25, n. 5, p. 454–460, May 2006.

PRUSKY, D.; KOBILER, I.; AKERMAN, M.; MIYARA, I. Effect of acidic solutions and acidic prochloraz on the control of postharvest decay caused by *Alternaria alternata* in mango and persimmon fruit. **Postharvest Biology and Technology**, Amsterdam, v. 42, n. 2, p. 134-141. Nov. 2006.

SCOTT, A. J.; KNOTT, M. A. A cluster analyses method for grouping means in the analyses of variance. **Biometrics**, Washington, v. 30, n. 3, p. 507-512, 1974.

SOYLU, E. M.; YIGITBAS, H.; TOK, F. M.; SOYLU, S.; KURT, S.; BAYSAL, O.; KAYA, A. D. Chemical composition and antifungal activity of the essential oil of *Artemisia annua* L. against foliar and soil-borne fungal pathogens. **Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz - Journal of Plant Diseases and Protection,** Stuttgart, v. 112, n. 3, p. 229-239, May 2005.

THANGAVELU, R.; SUNDARARAJU, P.; SATHIAMOORTHY, S. Management of anthracnose disease of banana caused by *Colletotrichum musae* using plant extracts. **Journal of Horticultural Science & Biotechnology,** Kent, v. 79, n. 4, p. 664-668, July 2004.

VICENT, A.; BADAL J.; ASENSI, M. J.; SANZ, N.; ARMENGOL, J.; GARC´IA-JIM´ENEZ, J. Laboratory evaluation of citrus cultivars susceptibility and influence of fruit size on Fortune mandarin to infection by *Alternaria alternata* pv. c*itri.* **European Journal of Plant Pathology**, Dordrech, v. 110**,** n. 3, p. 245–251, Mar. 2004.

CHAPTER 3

Potentiality of plant extracts to control of *Alternaria alternata* in Murcott tangor fruits

Daniel D.C. Carvalho [a], Eduardo Alves [a,] *, Denilson F. Oliveira [b], Renato B. Camargos [a] and Tereza R.S. Batista [c]

[a] *Departamento de Fitopatologia, Universidade Federal de Lavras, C.P.: 3037, 37200-000, Lavras - MG - Brazil.* [b] *Departamento de Química, Universidade Federal de Lavras, C.P.: 3037, 37200-000, Lavras - MG - Brazil.* [c] Centro *Universitário de Lavras, Rua Padre José Poggel, 506, Centenário, 37200-000, Lavras - MG - Brazil.*

_____

* Corresponding author. Phone: (55) (35) 3829-1789; fax: (55) (35) 3829-1283. *E-mail address*: ealves@ufla.br (E. Alves).

(Formatted according to *Postharvest Biology and Technology)*

Abstract

In order to select potentially helpful plant extracts for *in vivo* control of *Alternaria alternata* of Murcott tangor, five plant extracts were previously selected *in vitro* condition and evaluated by assay with Murcott tangor fruits. Plant material obtained from plants collected in Alto Rio Grande and São Francisco Rivers regions of Minas Gerais State, Brazil, was extracted with methanol solvent to obtain a solid plant extract. In relation to in *vivo* assay, plant extracts were dissolved in an *A. alternata* conidia suspension at 10^6 conidia mL^{-1} and applied on Murcott tangor fruits through perforations made with a needle. After 8 and 12 days, the diameters of Alternaria brown spots (ABS) were measured. Among the plant extracts evaluated, the one obtained from *Anadenanthera colubrina* was the most effective against the pathogen, which produced equal performance regarding the evaluated fungicides. The plant extract was able to cause near to 51% of suppression in the development of ABS on fruits with 12 days post inoculated, showing high potential to *in vivo A. alternata* control.

Keywords: Anadenanthera colubrina; Alternaria brown spot; Citrus.

Introduction

Alternaria brown spot (ABS), an important and serious disease of many tangerines and their hybrids in humid and semiarid areas around the world (Timmer et al., 2003), is caused by the fungus *Alternaria alternata* (Fr:Fr) Keissl f. sp. *citri*, which has been found in São Paulo and Minas Gerais States, in Brazil, bringing about economical losses to Murcott tangor producers (Peever et al., 2004; Prates, 2007). Such microorganism can attach both leaves and fruits, reducing production by plant. Besides, it causes lesions to fruits, turning them unmarketable (Vicent et al., 2000).

Consequently, foliar fungicide applications are usually necessary to produce fruit with good external quality in areas where ABS is common. Depending on climate, 3 to 15 applications may be necessary (Timmer et al., 2003), causing an increase in production cost and contamination of foods and the environment with toxic substances (McFayden, 1998). Thus, cheaper and less harmful products to control *A. alternata* in Murcott tangor are greatly welcome. Considering the ability of plants to make environmental friendly substances to control plant diseases (Thangavelu et al., 2004; Lindsey & Staden, 2006) and the previously detected *in vitro* antifungal activity by five plant extracts (Carvalho et al., 2007), this work aimed to contribute for the development of new methods to control *A. alternata* on Murcott tangor (probable *Citrus sinensis* (L.) Osb. x *C. reticulata* Blanco hybrid) by the evaluation of those plant extracts performance on Murcott tangor fruits inoculated with such fungus.

Materials and Methods

Preparation of plant extracts

Five plants (Table 1) previously selected by Carvalho et al. (2007) were collected next to Alto Rio Grande and São Francisco river regions of Minas Gerais State, Brazilian southeast region, and identified by comparison with specimens available in the Herbarium ESAL-Universidade Federal de Lavras. They were dried in an oven at 40°C during 48 h, grounded and exhaustively extracted with methanol at room temperature. The solvent present in the liquid phase was removed under vacuum to afford the extracts, which were stored at -10°C until the moment to be used.

Table 1. Plants collected in Alto Rio Grande and São Francisco river regions of Minas Gerais State, Brazil.

Plant extracts	Part of plant	Family	Region of collection*
Anadenanthera colubrina (Vell.) Brenan	Barks	Fabaceae	São Francisco
Artemisia annua L.	Leaves	Asteraceae	Alto Rio Grande
Ruta graveolens L.	Flowers and Leaves	Rutaceae	Alto Rio Grande
Cariniana estrellensis (Raddi) Kuntze	Barks	Lecythidaceae	São Francisco
Fícus carica L.	Leaves	Moraceae	Alto Rio Grande

Fungus isolation

Pieces (10 – 25 mm^2) of washed fruit barks from Murcott tangor were

subsequently immersed into 70% ethanol (30-60 s), 2% sodium hypoclorite (30-60 s) and distilled water (2 x 30 s). Four fragments were placed in a Petri dish containing potato-dextrose-agar (PDA) (200 g cooked potato, 20 g dextrose, 20 g agar and 1 L distilled water). After seven days at 25ºC, under a 12 h photoperiod, 9 mm agar plugs of the medium containing the fungus mycelium were transferred to new Petri dishes with the same culture medium (Prusky et al., 2006). Subsequently, *Alternaria alternata* (Fr:Fr) Keissl f. sp. *citri* was isolated and identified as described by Carvalho et al. (2007).

Assay with Murcott tangor fruits

To produce enough conidia, the fungus was grown on PDA during seven days, at 25ºC, under 12 h photoperiod provided by fluorescent light mixed with black fluorescent NUV lamp (Bóveda, 1986). Only ripe and healthy Murcott tangor fruits washed were used. They were dried during 60 min under aseptic conditions in a laminar flux chamber. Four points were selected around the point of their insertion with the plant and after, four perforations (3 mm deep) were made with a needle on each point selected. Then, adapting the procedure described by Dantas et al. (2003) and Colturato (2006), 2 mg of each extract were dissolved in 600 µL of an aqueous 10^6 conidia mL^{-1} suspension containing Tween 80 at 1% (g mL^{-1}), to result in mixtures from which 20 µL were collected and poured into perforations of each selected point. This experiment was carried out with four fruits per treatment, using the conidia suspension in Tween 80 as negative control and mixtures of conidia suspension and the fungicides Dacobre PM (chlorotalonil 250 g Kg^{-1} and copper oxychloride 504 g Kg^{-1}, produced by Iharabras S. A. Chemical Industries) at 3.5 mg mL^{-1} and Amistar (Azoxistrobin 500g Kg^{-1}, produced by Syngenta Crop Protection) at 0.16 mg mL^{-1} as positive controls. Four fruits were also treated with 1% Tween 80 (20 µL/point) containing no conidium, plant extract or fungicide. All fruits were kept in a

BOD at 25ºC, under 12 h of photoperiod. After 8 and 12 days, the lesion diameter around each inoculation point was measured with a rule. Average value of spots of each fruit was converted into spots development rate (ABS dr) before statistical analysis. For conversion, average value of each fruit was divided by the average value of all negative control fruits.

Statistical analysis

Converted data from treatments were submitted to analysis of variance and average values were compared by Scott-Knott (1974) calculations ($P \leq 0.05$). Statistical analyses were done using SISVAR software (Ferreira, 2000).

Results

Among the five plant extracts studied, only that from *Anadenanthera colubrina* afforded satisfactory suppression of lesions caused by *A. alternata* on tangor fruits (Table 2). Although a small efficiency was observed after a longer period of time (12 days), results obtained with such plant extract were statistically similar to the ones afforded by the commercial fungicides. A 16-day evaluation was also tried in this experiment but, except for fungicides and *A. colubrina* treatments, all inoculated surfaces of fruits were completely deteriorated by fusion of all lesions. No disease was observed on fruits treated with 1% Tween 80 solution containing no conidium.

Table 2. Effect of plant extracts and fungicides on Murcott tangor fruits inoculated with *Alternaria alternata*.

Treatments	ABS dr [a]		ABS dr [b]
	8 days	12 days	
Anadenanthera colubrina	0.3884 aA	0.4914 aB	49%
Artemisia annua	1.1140 bA	1.0990 bA	109%
Ruta graveolens	1.0072 bB	0.8638 bA	86%
Cariniana estrellensis	0.8923 bA	1.0272 bB	103%
Ficus carica	0.9443 bA	0.9808 bA	98%
Chlorotalonyl and Copper oxychloride [c]	0.6336 aA	0.5751 aA	58%
Azoxistrobin [d]	0.6110 aA	0.7091 aB	71%
1% Tween 80 [e]	1.0000 bA	1.0000 bA	100%
Coefficient of variability	22.43%	21.81%	

[a] Means with the same small letter in a column and capital letter in a line do not differ significantly ($P \leq 0.05$) according to the Scott-Knott (1974) calculations; [b] ABS dr from fruits with 12 days converted into percentage; [c] 0.87 and 1.76 g L^{-1}, respectively; [d] 0.08 g L^{-1}; [e] 10^6 conidia mL^{-1} suspensed with 1% Tween 80.

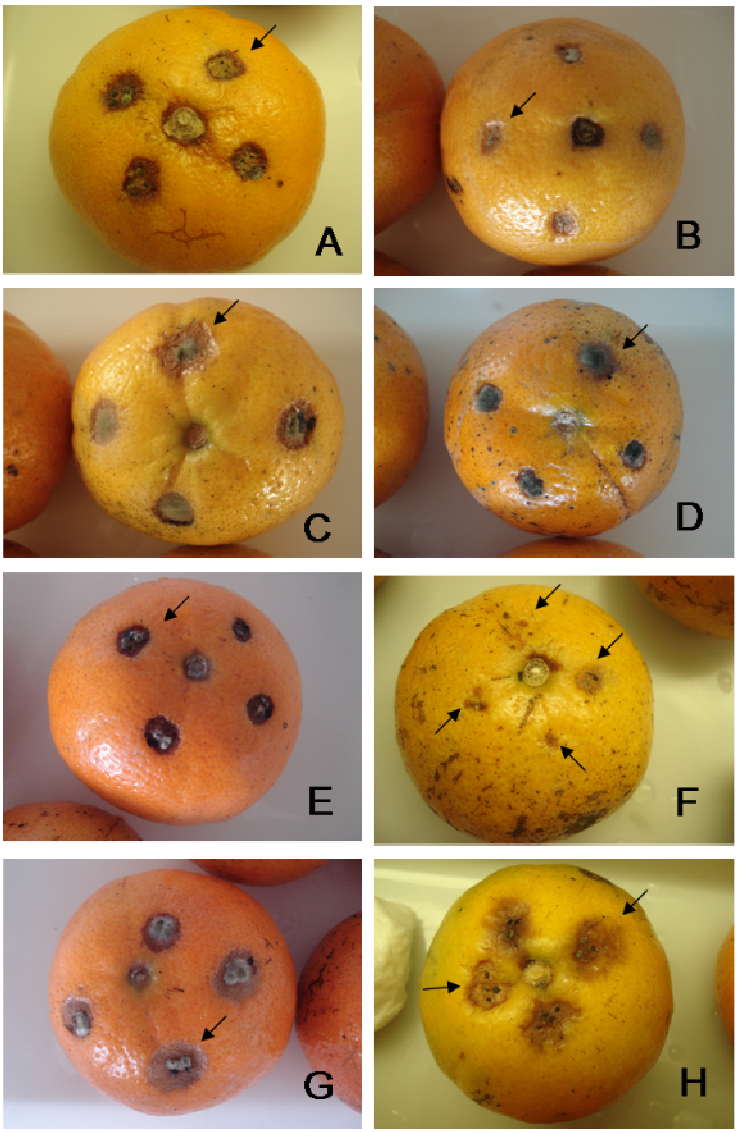

Fig. 1. Effect of plant extracts and fungicides on Alternaria brown spots in Murcott tangor fruits eight days after inoculation with 10^6 *Alternaria alternate* conidia mL^{-1}. (A) Chlorotalonyl and Copper oxychloride at 0.87 and 1.76 g L^{-1}, respectively; (B) Azoxistrobin at 0.08 g L^{-1}; (C) *F. carica*, (D) *C. estrellensis*, (E) *R. graveolens*, (F) *A. colubrina* and (G) *A. annua* extract at 3.33 g L^{-1}; (H) 10^6 conidia mL^{-1} suspended with 1% Tween 80.

Discussion

Anadenanthera colubrina appears to be a very promising source of new products to control *Alternaria alternata* in citrus, since the extract of such plant afforded values statistically similar to those observed for the commercial fungicides (Table 2). Although medicinal properties such as immunomodulatory and anti-tumoral activities have already been described for *A. colubrina* (Moretao et al., 2004), no previous report about its ability to produce antifungal substances was found in the literature.

To exemplify the known antifungal property of *A. annua,* Soylu et al. (2005) verified a chemical composition of its essential oil, which was determined by GC/MS analysis. They also verified antifungal activity of the essential oil in an *in vitro* condition. The effect of the essential oil was evaluated against economically important foliar and soil-borne fungal pathogens of tomato, including *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Phytophthora infestans* and *Verticillium dahliae*. A complete inhibition of the conidial germination and germ tube elongation of both fungal pathogen tested occurred. Besides, it was found in the pertinent literature new report concerning essential oil of *Artemisia argyi* inflorescence exhibiting antifungal activity against *Botrytis cinerea* and *A. alternata*, whereas the inhibition of *B. cinerea* and *A. alternata* were 93.3 and 84.7% for oil extracted by hydrodistillation, when exposed to a concentration of 1 mg mL^{-1} (Guan et al., 2006). Arteannuin B, a main sesquiterpenoid in *A. annua*, showed antifungal activity against *Gaeumannomyces graminis*, *Rhizoctonia cerealis*, *Gerlachia nivalis* and *Verticillium dahliae*, with minimum inhibitory capacity (MIC) of 0.15, 0.10, 0.15 and 0.10 mg mL^{-1}, respectively (Tang et al., 2000). The concentration of gross extract from *A. colubrina* in this work (3.33 mg mL^{-1}) can provide values as low as verified to Guan et al. (2006) and Tang et al. (2000), after purification of active fractions. Besides, extract from *A. annua* used in this study can produce

small quantity of oil when compared with essential oil studied by Soylu et al. (2005). This explanation can justify the inactivity of *A. annua* extract on *A. alternata* (Table 2).

It was not expected a low *in vivo* effect on ABS for *Ruta graveolens* and *Ficus carica* extracts, once its activity on plant pathogenic fungi is known (Aqil & Ahmad, 2003; Meepagala et al., 2005).

Perhaps *A. annua, R. graveolens, F. carica* and *C. estrellensis* extracts do not have a later effect, required for *in vivo* assay (8 at 12 days), or could be less toxic to *A. alternata* than *A. colubrina* extract when used for *in vivo* control, even in contact with fungus conidia.

Interestingly, *A. colubriana* produced an excellent result with 8 as well as 12 days, especially in respect to fungicides. Copper fungicides are widely used for controlling Alternaria in Florida and, when applied on a timely basis, provide good control of the disease and azoxystrobin is generally more effective than trifloxystrobin (Timmer et al., 2003). Azoxystrobin has been recently used with success on citrus orchards of Minas Gerias state, Brazil. The use of *A. colubrina* extract must be explored, once strobilurins are single site of action fungicides (Sierotzki et al., 2000) and thus, prone to resistance development and must be alternated or mixed with other products (Timmer et al., 2003).

A possible success of alternative control in the orchards can rely on field conditions. Lesions on fruit appear to require more time to produce conidia than lesions on the leaves (Reis & Goes, 2005). Furthermore, Whiteside (1998) noted in fields that sporulation was more abundant on leaves than on fruit. A suggestion for future studies is based on the application of plant extract on fruits, close to harvest season, aimed to reduce levels of agrochemicals in the fruits and to obtain a better performance of control. This practice would be rotated with chemical control, whereas the residual time could be 12 days to *A. colubrina* extract.

According to the found and discussed results, it is possible to conclude that *A. colubrina* presents great potential to *in vivo* control of *A. alternata* in Murcott tangor fruits. Thus, future studies must be carried out to evaluate the extract effect and residual time under field conditions.

Acknowledgements

The authors gratefully acknowledge CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for a master fellowship and Dr. Paulo César Lima (Departamento de Ciências Exatas – Universidade Federal de Lavras) for support in statistical analysis. This work was financially supported by CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior).

References

AQIL, F.; AHMAD, I. Broad-spectrum antibacterial and antifungal properties of certain traditionally used Indian medicinal plants. **World Journal of Microbiology & Biotechnology,** Dordrecht, v. 19, p. 653-657, 2003.

BÓVEDA, R. R. M. **Morfologia, patogenicidade, esporulação e sensibilidade a fungicidas *in vitro* de *Alternaria solani* (Ell. e Mart.) Jones e Grout e *Alternaria alternata* (Fr.) Keissler de solanáceas.** 1986. 106 p. Dissertação (Mestrado em Fitopatologia) – Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, SP.

CARVALHO, D. D. C.; CAMARGOS, R. B.; ALVES, E.; BATISTA, T. R. S.; SILVA, R. C.; OLIVEIRA, D. F. Bioensaios para a identificação de extratos vegetais com propriedades fungitóxicas. In: CONGRESSO DE PÓS-GRADUAÇÃO DA UFLA, 16., 2007, Lavras. **Resumos...** Lavras: APG, 2007. 1 CD-ROM.

COLTURATO, A. B. **Efeito do meio de cultura, temperatura, fotoperíodo e fungicidas no crescimento micelial e no controle de *Alternaria alternata* f. sp. *citri*, causador da mancha marrom do tangor Murcote.** 2006. 53 p. Dissertação (Mestrado em Proteção de Plantas) - Universidade Estadual Paulista - Faculdade de Ciências Agronômicas, Botucatu, SP.

DANTAS, S. A. F.; OLIVEIRA, S. M. A.; MICHEREFF, S. J.; NASCIMENTO, L. C.; GURGEL, L. M. S.; PESSOA, W. R. L. S. Doenças fúngicas pós-colheita em mamões e laranjas comercializados na Central de Abastecimento do Recife. **Fitopatologia Brasileira**, Brasília, v. 28, n. 5, p. 528-533, set./out. 2003.

FERREIRA, D. F. Análises estatísticas por meio do Sisvar para Windows versão 4.0. In: REUNIÃO ANUAL DA REGIÃO BRASILEIRA DA SOCIEDADE INTERNACIONAL DE BIOMETRIA, 45., 2000, São Carlos. **Anais...** São Carlos: Universidade Federal de São Carlos, 2000. p. 255-258.

GUAN, W. Q.; LI, S. F.; YAN, R. X.; HUANG, Y. F. Comparison of composition and antifungal activity of *Artemisia argyi* Levl. et Vant inflorescence essential oil extracted by hydrodistillation and supercritical carbon dioxide. **Natural Product Research**, Oxon, v. 20, n. 11, p. 992-998, Sept. 2006.

LINDSEY, K. L.; STADEN, J. V. Growth inhibition of plant pathogenic fungi by extracts of *Allium sativum* and *Tulbaghia violacea*. **South African Journal of Botany,** Grahamstown, v. 70, n. 4, p. 671-673, Oct. 2004.

McFAYDEN, R. E. C. Biological control of weeds. **Annual Review of Entomology,** Palo Alto, v. 43, p. 369-393, 1998.

MEEPAGALA, K. A.; SCHRADER, K. K.; WEDGE, D. E.; DUKE, S. O. Algicidal and antifungal compounds from the roots of *Ruta graveolens* and synthesis of their analogs. **Phytochemistry**, Oxford, v. 66, n. 22, p. 2689-2695, Nov. 2005.

MORETAO, M. P.; ZAMPRONIO, A. R.; GORIN, P. A. J.; IACOMINI, M.; OLIVEIRA, M. B. M. Induction of secretory and tumoricidal activities in peritoneal macrophages activated by an acidic heteropolysaccharide (ARAGAL) from the gum of *Anadenanthera colubrina* (Angico branco). **Immunology Letters**, Amsterdan, v. 93, n. 2-3, p. 189-197, May 2004.

PEEVER, T. L.; SU, G.; CARPENTER-BOGGS, L.; TIMMER, L. W. Molecular systematics of citrus-associated *Alternaria* spp. **Mycologia,** Teheran, v. 96, n. 1, p. 119-134, Jan./Feb. 2004.

PRATES, H. S. **Mancha de alternaria das tangerinas**. Campinas: Secretaria de Agricultura e Abastecimento, 2007. 4p. Disponível em: <http://www.cati.sp.gov.br/novacati/tecnologias/doencas_de_plantas/alternaria/mancha_alternaria.htm>. Acesso em: 27 fev. 2007.

PRUSKY, D.; KOBILER, I.; AKERMAN, M.; MIYARA, I. Effect of acidic solutions and acidic prochloraz on the control of postharvest decay caused by *Alternaria alternata* in mango and persimmon fruit. **Postharvest Biology and Technology**, Amsterdam, v. 42, n. 2, p. 134-141, Nov. 2006.

REIS, R. F.; de GOES, A. Effect of lesion age, humidity, and fungicide application on sporulation of *Alternaria alternata*, the cause of brown spot of tangerine. **Plant Disease**, Saint Paul, v. 90, n. 8, p. 1051-1054, Aug. 2005.

SCOTT, A. J.; KNOTT, M. A. A cluster analyses method for grouping means in the analyses of variance. **Biometrics**, Washington, v. 30, n. 3, p. 507-512, 1974.

SIEROTZKI, H.; PARISI, S.; STEINFELD, U.; TENZER, I.; POIREY, S.; GISI, U. Mode of resistance to respiration inhibitors at the cytochrome bc(1)

enzyme complex of *Mycosphaerella fijiensis* field isolates. **Pest Management Science**, Sussex, v. 56, n. 10, p. 833-841, Oct. 2000.

SOYLU, E. M.; YIGITBAS, H.; TOK, F. M.; SOYLU, S.; KURT, S.; BAYSAL, O.; KAYA, A. D. Chemical composition and antifungal activity of the essential oil of *Artemisia annua* L. against foliar and soil-borne fungal pathogens. **Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz - Journal of Plant Diseases and Protection,** Stuttgart, v. 112, n. 3, p. 229-239, May 2005.

TANG, H. Q.; HU, J.; YANG, L.; TAN, R. X. Terpenoids and flavonoids from Artemisia species. **Planta Medica**, Stuttgart, v. 66, n. 4, p. 391-393, May 2000.

THANGAVELU, R.; SUNDARARAJU, P.; SATHIAMOORTHY, S. Management of anthracnose disease of banana caused by *Colletotrichum musae* using plant extracts. **Journal of Horticultural Science & Biotechnology,** Kent, v. 79, n. 4, p. 664-668, July 2004.

TIMMER, L. W.; PEEVER, T. L.; SOLEL, Z.; AKIMITSU, K. Alternaria diseases of citrus - novel pathosystems. **Phytopathologia Mediterranea,** Florence, v. 42, n. 2, p. 99–112, Aug. 2003.

VICENT, A.; ARMENGOL, J.; SALES, R.; GARC´IA-JIM´ENEZ, J.; ALFARO-LASSALA, F. First report of alternaria brown spot of citrus in Spain. **Plant Disease**, Saint Paul, v. 84, n. 9, p. 1044, Sept. 2000.

WHITESIDE, J. O. Alternaria brown spot of mandarins. In: WHITESIDE, J. O.; GARNSEY, S. M.; TIMMER, L. M. **Compendium of citrus diseases**. Saint Paul: American Phytopatological Society, 1988. p. 8.

Final considerations

Alternaria brown spot (ABS) was found in many regions of the São Paulo and Minas Gerais states, causing injury to Ponkan tangerine and Murcott tangor fruits, rendering them unmarketable. The methods for controlling ABS are based on the use of fungicides, which increase production costs, contaminate foods and the environment with toxic substances. Besides, those substances have not produced the required efficiency to control *Alternaria alternata* on fruits. Thus, cheaper, more efficient and less toxic products active against such fungus are greatly welcome, given that plants are considered sources of environmental friendly substances to control plant diseases. This work can be considered an important contribution to control *A. alternata* from citrus, given that *Anadenanthera colubrina* extract was applied on fruits, resulting in a meaningful control obtained after 8 and 12 days. This methodology can reduce levels of agrochemicals in the fruits and improving the control performance both in fields and post harvest conditions. In future studies the extract from *A. colubrina* must be purified and its activity verified on fruits in field conditions and in postharvest conservation.