ANTIFUNGAL ACTIVITY AND ULTRASTRUCTURAL ALTERATIONS IN *Pseudocercospora griseola* TREATED WITH ESSENTIAL OILS

Atividade antifúngica e alterações ultraestruturais em *Pseudocercospora griseola* tratado com óleos essenciais

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ABSTRACT

Pseudocercospora griseola, the etiologic agent of angular leaf spot of common bean (*Phaseolus vulgaris*), is an important disease in all bean-producing regions worldwide and may cause extremely high yield losses. The control of this disease is made more difficult by the pathogen's genetic variability and the inefficiency of fungicides. In this study, of 26 essential oils tested at different concentrations, 25 demonstrated efficiency in affecting the germination of strains 63-31 and 63-63 of the pathogen, reaching inhibition levels of between 80% and 100%. *Cymbopogon citratus* and *Cymbopogon martinii* inhibited conidia germination at all concentrations; *Eugenia caryophyllata*, *Cinnamomum* sp., *Thymus vulgaris*, *Matricaria recutita*, *Cordia verbenacea*, *Origanum vulgare*, *Cymbopogon nardus*, at 0.1 and 0.5%; and *Zingiber officinale*, *Mentha arvensis*, *Chamaecyparis pisifera*, *Lavandula officinalis*, *Ocimum basilicum*, *Pimpinella anisum*, *Ocimum selloi*, *Baccharis dracunculifolia*, *Laurus nobilis*, *Citrus sinensis*, *Melaleuca alternifolia* and *Eucalyptus globulus*, at 0.5%. The main constituents identified were cinnamaldehyde in *Cinnamomum* sp.; eugenol in *E. caryophyllata*; *trans*-β-farnesene in *M. recutita*; pulegone in *C. verbenacea*; thymol in *T. vulgaris*; geranial and neral in *C. citratus*, and geraniol in *C. martini*. Through transmission electron microscopy (TEM), it was verified that *C. citratus*, *C. martini* and *E. caryophyllata* presented direct fungitoxic action on *P. griseola*, causing severe damage to the cellular ultrastructure of the conidia, invalidating germination. These results indicated that essential oils are a promising alternative strategy for the control of angular leaf spot in bean, representing less risk to human health and the environment.

Index terms: Angular leaf spot, transmission electron microscopy, alternative control of plant disease.

RESUMO

Pseudocercospora griseola, agente etiológico da mancha angular do feijoeiro comum (*Phaseolus vulgaris*), é uma doença importante nas regiões produtoras de feijão em todo o mundo e pode causar perdas de produtividade extremamente elevados. O controle dessa doença é dificultado pela variabilidade genética do patógeno e da ineficiência de fungicidas. Neste estudo, de 26 óleos essenciais testados em concentrações diferentes, 25 demonstraram eficiência em inibir a germinação das linhagens 63-31 e 63-63 do agente patogênico, atingindo níveis de inibição entre 80% e 100%. *Cymbopogon citratus* e *Cymbopogon martini* inibiram a germinação de conídios em todas as concentrações; *Eugenia caryophyllata*, *Cinnamomum* sp., *Thymus vulgaris*, *Matricaria recutita*, *Cordia verbenacea*, *Origanum vulgare*, *Cymbopogon nardus*, em 0,1 e 0,5%, e *Zingiber officinale*, *Mentha arvensis*, *Chamaecyparis pisifera*, *Lavandula officinalis*, *Ocimum basilicum*, *Pimpinella anisum*, *Ocimum selloi*, *Baccharis dracunculifolia*, *Laurus nobilis*, *Citrus sinensis*, *Melaleuca alternifolia e Eucalyptus globulus*, em 0,5%. Os principais constituintes identificados foram cinamaldeído em *Cinnamomum* sp.; Eugenol em *E. caryophyllata*; trans-β-farneseno em *M. recutita*; pulegona em *C. verbenacea*; timol em *T. vulgaris*; geranial e neral em *C. citratus* e geraniol em *C. martini*. Através de microscopia eletrônica de transmissão (TEM), verificou-se que *C. citratus*, *C. martini* e *E. caryophyllata* apresentaram ação antifúngica direta sobre *P. griseola*, causando graves danos na ultraestrutura celular dos conídios, invalidando a germinação. Esses resultados indicaram que os óleos essenciais são uma estratégia alternativa promissora para o controle da mancha angular do feijoeiro, o que representa menos risco para a saúde humana e ao ambiente.

Termos para indexação: Mancha angular, microscopia eletrônica de transmissão, controle alternativo de doenças de plantas.

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INTRODUCTION

Angular leaf spot (ALS), caused by *Pseudocercospora griseola* (Sacc.) Crous & U. Braun, is the most widespread disease in all bean-producing regions worldwide. According to the Commonwealth Mycological Institute (CMI), this disease occurs in more than 60 countries (SARTORATO; RAVA, 1994) and, under favorable environmental conditions, yield losses may reach 80% (JESUS-JÚNIOR et al., 2001; STENGLEIN et al., 2003; PAULA-JÚNIOR; ZAMBOLIM, 2006). Although application of fungicides is the primary measure taken to

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control plant diseases, their continuous and indiscriminate use has caused contamination by waste, environmental pollution and the selection of resistant populations of pathogens. Chemical control carries considerable environmental and financial costs (MIKLAS et al., 2006). Due to the high genetic variability of *P. griseola* (BROCK, 1951; PYNDJI, 1993; PASTOR-CORRALES et al., 1998; OROZCO; ARAYA, 2005; STENGLEIN; BALATTI, 2006; SILVA, et al., 2008), genetic control through the use of resistant cultivars is very difficult.

The inefficiency of pesticides in the control of ALS and the lack of resistant cultivars, as well as market demands in food production and the need to achieve environmental sustainability, food security and economic viability, have all made the use of technology that is non-aggressive to the environment and human health into a major challenge for bean producers. Considering these problems, it has become vital to develop strategies based on the rational use of fungicides or to replace them with alternative products (CHRISTIAN; GOGGI, 2008; GHINI; KIMATI, 2000). Essential oils have shown biological activity that may offer an alternative strategy in the control of plant pathogens (MIHALIAK et al., 1991; STANGARLIN et al., 1999; ISMAN, 2000; KOTZEKIDOU et al., 2008) due to fungitoxic action (FUORI et al., 2000; BONALDO et al., 2004; BANG, 2007) or by inducing resistance in plants (SCHWAN-ESTRADA, 2003; QUINTANILLA et al., 2002; PEREIRA et al., 2011).

The essential oils extracted from medicinal, aromatic and ornamental plants have shown efficiency in controlling plant pathogens (SHAHI et al., 2003; SOYLU et al., 2006; LEE et al., 2007; BARRERA-NECHA et al., 2008). The effects of essential oils have been studied at the ultrastructural level for some pathogens (ZAMBONELLI et al., 1996; RASOOLI et al., 2006; ROZWALKA et al., 2010). However, there are no reports about the antifungal activity and the mode of action of essential oils on *P. griseola*. This study aimed to evaluate (i) the antifungal activity of 26 essential oils on germination of *P. griseola* conidia, strains 63-31 and 63-63; (ii) the effect of the essential oils of *Cymbopogon martini*, *Eugenia caryophyllata* and *Cymbopogon citratus* on conidia at ultrastructural level by transmission electron microscopy (TEM), and (iii) to identify the chemical constituents of essential oils with fungitoxic activity against this plant pathogen.

MATERIALS AND METHODS

Fungal isolates

Two monosporic isolates of *P. griseola* were employed, classified as pathotypes 63-31 and 63-63, from the mycology collection of the Institute of Biotechnology applied to Agriculture (BIOAGRO) at the *Federal University of* Viçosa (Viçosa, Minas Gerais State, Brazil). Conidia were obtained by culturing the fungus on bean leaf-dextrose-agar medium in a growth chamber (Fanem, São Paulo, Brazil) at 24 ± 2º C. After approximately 15 days, spore suspension was prepared by adding 5–10 mL of sterile distilled water to each Petri dish and scraping the surface of the culture with a spatula.

Essential oils

The essential oils extracted from 26 medicinal, ornamental, aromatic, or forest species representative of the families Apiaceae (*Pimpinella anisum* L.), Asteraceae (*Baccharis dracunculifolia* DC., *Matricaria recutita* L.), Boraginaceae (*Cordia verbenacea* DC.), Cupressaceae (*Chamaecyparis pisifera* (Siebold & Zucc.) Endl., *Chamaecyparis plumosa* Hort. ex Beissn.), Lamiaceae (*Lavandula officinalis* Chaix *ex* Vill., *Mentha arvensis* L., *Ocimum basilicum* L., *Ocimum selloi* Benth., *Origanum vulgare* L., *Rosmarinus officinalis L.*, *Thymus vulgaris* L.), Lauraceae (*Cinnamomum* sp., *Laurus nobilis* L.), Myrtaceae (*Corymbia citriodora* (Hook.) K.D. Hill & L.A.S. Johnson, *Eucalyptus globulus* Labill., *Eugenia caryophyllata* Thunb., *Melaleuca alternifólia* Cheel), Poaceae (*Cymbopogon citratus* (DC) Stapf., *Cymbopogon martini* (Roxb.) Wats., *Cymbopogon nardus* (L.) Rendle), Rutaceae (*Citrus limon* (L.) Burm.f., *Citrus sinensis* (L.) Osbeck.), Verbenaceae (*Lippia citriodora* Kunth) e Zingiberaceae (*Zingiber officinale* Roscoe).

Essential oils from *L. officinalis*, *O. selloi*, *O. vulgare* and *Cinnamomum* sp. were extracted by hydrodistillation for 3 h in a Clevenger-type apparatus. Flowers and leaves from *L. officinalis*, and leaves from *O. selloi* and *O. vulgare* were collected at the Medicinal Plants Nursery of the Agricultural Department, Federal University of Lavras, Minas Gerais State, Brazil, at vegetative stage, and cinnamon bark was purchased in a natural store in Lavras city. The remaining essential oils were supplied by Chamel Industry and Natural Products Commerce, Paraná State, Brazil (2010). All essential oils used in this work were kept in amber flasks and stored under refrigeration at 0º C.

Screening the essential oils with *in vitro* antifungal activity

A total of 40 µL of essential oils at 0.04%, 0.2% and 1.0% and 40 µL of aqueous conidial suspension (2 x 10^4 conidia.mL^{-1}) prepared from 15-day-old cultures of strains 63-31 and 63-63 were placed inside each well of a

96-well plate. This mixture resulted in the final concentrations at 0.02% (200 $\mu L.L^{-1}$), 0.1% (1000 $\mu L.L^{-1}$) and 0.5% (5000 $\mu L.L^{-1}$). These treatments were prepared using sterile distilled water with Tween 20 at 1.0%. Sterile distilled water (40 µL) mixed with conidial suspension was used as control. In previous germinations tests comparing germination in water and water plus Tween-20 a 1.0%, it was observed that Tween showed non-toxic effects in both strains of pathogen. After 20 hours of incubation at 24 ± 2º C and 12 h photoperiod, germination was interrupted by addition of 20 µL of lactoglycerol cotton blue. The percentage of germination was estimated by counting 50 randomly chosen conidia in 6 wells, totaling 300 conidia per treatment, under a light microscope. Conidia presenting differentiated germ tubes, independently of their size, were considered germinated. The experiment were carried out in a completely randomized design, as a factorial 2 x 3 x 3 (strains x oil x concentrations) x 6 repetitions. Each well of the plate was an experimental unit.

Chemical composition of essential oils

C. martini (0.144 mg), *M. chamomilla* (0.127 mg), *E. caryophyllata* (0.200 mg), *T. vulgaris* (0.147 mg), *C. verbenacea* (0.179 mg), *C. citratus* (0.160 mg) and *Cinnamomum* sp. (0.118 mg) were dissolved in 0.5 mL of dichloromethane (CH_2Cl_2). These oils showed antifungal activity of more than 90%, when tested at 0.1%. One microliter of diluted essential oils was injected in gas chromatography coupled with mass spectrometry (GCMS-QP2010 Plus, Shimadzu). The gas chromatography operated with ultra pure helium as the carrier gas at a flow rate of 1.8 $mL.min^{-1}$ and the separation was done on an Equity-5 capillary column (30 m x 0.22 mm I.D with 0.25 µm film thickness), with column oven at 60º C for 2 min, heating at 3º $C.min^{-1}$ up to 240º C, remaining at 240º C for 15 min. The temperature of the injector was 220º C, split injection 1:15, manual injection, time run of 77 min. The mass spectrometry used a GC-MS interface, at 250º C, with ion source at 200º C and electron impact ionization at 70 eV. The compounds in essential oils were identified by comparing the similarity index of the mass spectra of relative area (%) with the data available in the library (Wiley 8) of GCMS.

Transmission electron microscopy (TEM) preparation

A conidial suspension (2 x 10^4 $conidia.mL^{-1}$) of strains 63-31 and 63-63, prepared in sterile distilled water with Tween 20 at 1.0%, was mixed with 0.5 mL of the solutions of 0.2% essential oils of *C. martini*, *E. caryophyllata* and *C. citratus* to obtain a final concentration of 0.1%. For the control, 0.5 mL conidial suspensions were mixed with 0.5 mL of sterile distilled water. The treatments remained under agitation in an Orbital Shaker at 100 rpm and an average temperature of 25 ± 2º C and, after 24 h, were centrifuged for 3 min at 6000 rpm, forming conidial masses. The masses were fixed in modified Karnovsky solution (glutaraldehyde at 2.5%, formaldehyde at 2.5% in sodium cacodylate buffer at 0.05 M, pH 7.2, and calcium chloride at 0.1 M) and kept in the refrigerator for 24 h (primary fixation). To form pellets, the conidial masses fixed in Karnovsky were centrifuged for 3 min at 6000 rpm. The supernatant was discarded, and the agarose gel at 1.0% was heated to about 45º C and mixed with the pellets. The blocks of agarose formed were washed three times for 10 min in sodium cacodylate buffer at 0.05 M, for post-fixation in osmium tetroxide at 2% in the hood. After a 4 h period, the blocks were washed three times in distilled water and submitted to contrast *en bloc* in an aqueous solution of uranyl acetate at 0.5% for one night in the refrigerator. The dehydration was done in a graded acetone series at concentrations of 25, 50, 75, 90 and 100% for 10 min each, except at 100%, which was dehydrated 3 times for 10 min each time.

Subsequently, the blocks were submitted to the embedding process, with the acetone being replaced by resin in an increasing gradient, remaining 8 h in Spurr resin (30%) and acetone (70%), 8 h in Spurr resin (70%) and acetone (30%) and twice for 24 h in pure Spurr resin (100%) at room temperature. Samples were transferred to silicone molds, prepared in advance and containing half of the volume with polymerized Spurr resin (100%), and covered with pure Spurr resin; these then remained in an oven at 70º C for 48 h for polymerization. The blocks were trimmed with razor blades to remove the excess resin and to obtain a trapezoidal shape. Semithin sections (0.5 mm) were made on a Reichert-Jung (Ultracut E) ultramicrotome with a glass knife, collected with a gold ring and placed on glass slides. These were then dried on a metal plate at about 60º C, covered with toluidine blue stain, heated on a metal plate until the formation of a gold border, washed with distilled water, dried on a hot plate and visualized by light microscopy for localization of fungal structures of interest and orientation of the ultrathin sections. The ultrathin sections (> 100 nm) were made with a diamond knife, collected on copper grids (300 mesh) previously coated with Formvar film, post-contrasted with aqueous solution of uranyl acetate (2%) and lead citrate (3%) (Reynolds, 1963) for 3 min on each, and washed with

distilled water. For each treatment, the test was repeated twice and at least two blocks per replicate were examined. The observation of the samples and the recording of the images were performed in a Zeiss EM 109 transmission electron microscope operating at 80 kv.

Statistical analysis

Data analysis was done using generalized linear models (GLMs), assuming a binomial distribution logit link function. The linear predictor was represented by $\log [p_i / (1- p_i)] = \beta_{0i} + \beta_{1i}. \sqrt{c}$, where p_i = probability of germination inhibition of conidia treated with essential oil i (i = 1, 2, …,26) at concentration c (c = 0.02, 0.1 and 0.5%) and β_{0i} and β_{1i} are parameters that bring form to a relationship. The inferences were conducted considering the parameter of overdispersion (ϕ) estimated by Pearson residues. All analyzes were performed in R environment (2.15.0) for statistical computing R Development Core Team, 2012.

RESULTS AND DISCUSSION

Antifungal activity of essential oils on germination of *P. griseola* strains, *in vitro*

The results of analysis of deviance showed significant interaction between treatments and concentrations of the essential oils, indicating that differences in the percentage of germination occurred in function of the essential oil and concentrations used. So studies were done separately for each pathogen strain. The antifungal activity of essential oils in the germination of *P. griseola* strains, expressed by the percentage of conidial germination inhibition, was calculated based on average number of conidia that germinated from each strain, 63-31 (46.67) and 63-63 (31.75) and the untreated control (Table 1).

Essential oils of *C. citratus*, *C. martini* and *E. caryophyllata* were more effective than the other treatments, showing at least 98% inhibition of conidial germination of strains 63-31 and 63-63 of *P. griseola* at all tested concentrations. At 0.1%, *C. citratus*, *C. martini*, *E. caryophyllata*, *Cinnamomum* sp., *T. vulgaris*, *M. recutita* and *C. verbenacea* also showed antifungal activity against both strains. The average germination inhibition values were higher than 93.57%. At 0.02%, conidia germination of strain 63-63 was almost totally inhibited by *C. citratus*, *C. martini*, *E. caryophyllata* and *Cinnamomum* sp. However, these two last essential oils did not show the same efficiency against strain 63-31, for which they only reduced inhibition to approximately 50%. A difference in inhibition response of the two pathogen strains was also observed more effectively on conidia treated with *M. arvensis*, *Z. officinale*, *C. pisifera*, *L. officinalis* and *B. dracunculifolia* at 0.1% and *C. plumosa*, *R. officinalis* and *L. citriodora* at 0.5%. The regression model allows us to observe these differences (Figure 1).

It is also possible to observe that increasing the concentration of essential oil of *C. citriodora* and *C. limon* did not result in greater inhibition of the pathogen strains. At 0.5%, of the 26 essential oils screened, 21 promoted inhibition of conidial germination between 86 to 100% for both strains of the pathogen (Table 2).

At all the tested concentrations, some essential oils stimulated conidial germination of both strains, expressed by the negative sign. *C. nardus* (0.02%), *Z. officinale* (0.02%), *R. officinalis* (0.1%) and *C. limon* (0.1%, 0.02% and 0.5%) were the essential oils that most stimulated conidial germination of strain 63-63 (Table 1). Based on the estimated coefficients of the logistic regression model, by increasing the concentration of *E. caryophyllata* and *Cinnamomum* sp. from 0.02 to 0.03 and 0.06%, *M. arvensis*, *Z. officinale*, *O. vulgare* and *C. nardus* from 0.1 to 0.41, 0.27, 0.13 and 0.14%, respectively, the conidial germination inhibition of strain 63-31 could be 95%. In this study, it was observed that increasing the concentration of most essential oils promoted their enhanced antifungal activity on *P. griseola* strains. However, the inhibitory effect promoted by essential oils was different for the two strains of the pathogen. Greater conidial germination inhibition was observed for strain 63-63, which is reported to be more aggressive than 63-31 in bean production in Brazil (NIETSCHE et al., 2001; DAMASCENO-SILVA et al., 2008).

Chemical composition of essential oils

Identification and quantification of the main chemical constituents of essential oils can be seen in table 3.

The main constituents identified were cinnamaldehyde (92.36%) in *Cinnamomum* sp.; eugenol (91.94%) in *E. caryophyllata*; geraniol (75.92%) in *C. martini*; pulegone (68.96%) in *C. verbenacea*; geranial (58.89%) followed by neral (38.50%) in *C. citratus;* thymol (33.72%) in *T. vulgaris* and trans-β-farnesene (31.17%) in *M. recutita.*

The cytotoxic activity of essential oils in general is mostly due to the presence of phenols, aldehydes and alcohols (SACCHETTI et al., 2005). This fact was corroborated in the present study, which identified the major components of the three essential oils that

presented most inhibition of germination. These were geranial and neral (aldehyde) in *C. citratus*; eugenol (phenol) in *E. caryophyllata* and geraniol (alcohol) in *C. martini*. In the literature, only the main compounds of some other essential oils, including eugenol, thymol, carvacrol, cinnamaldehyde, inter alia, have been studied. The minor constituents of the essential oils studied here could also have a relatively strong antimicrobial effect (BURT, 2004; BAKKALI et al., 2008), which can indicate that these molecules have synergism in antimicrobial activity. The synergistic effects of the diversity of major and minor compounds of the essential oils should be taken into consideration to account for their antimicrobial activity.

Table 1 – Inhibition of conidial germination of *Pseudocercospora griseola* strains 63-31 and 63-63 by essential oils tested at 0.5%, 0.1% and 0.02%, *in vitro*.

Essential oils	Concentrations (%)					
	0.5		0.1		0.02	
	Strains		Strains		Strains	
	63-31	63-63	63-31	63-63	63-31	63-63
Cymbopogon citratus	100.00	100.00	99.64	100.00	100.00	100.00
Cymbopogon martini	99.29	100.00	98.93	100.00	99.64	99.48
Eugenia caryophyllata	100.00	100.00	100.00	100.00	58.21	99.48
Cinnamomum sp.	100.00	100.00	98.93	99.48	51.79	98.43
Thymus vulgaris	98.21	100.00	98.93	100.00	17.50	2.36
Matricaria recutita	100.00	100.00	93.57	100.00	3.57	13.91
Cordia verbenacea	98.93	99.48	94.64	96.33	4.29	10.24
Origanum vulgare	100.00	100.00	86.79	87.40	0.36	11.81
Cymbopogon nardus	100.00	100.00	83.21	89.50	6.79	-36.48
Zingiber officinale	99.29	100.00	50.71	95.28	1.79	-35.43
Mentha arvensis	99.64	100.00	35.71	99.48	14.29	59.58
Chamaecyparis pisifera	100.00	100.00	21.79	78.48	-0.71	25.98
Lavandula officinalis	100.00	100.00	16.07	80.05	1.43	61.15
Ocimum basilicum	97.86	100.00	26.79	57.48	26.79	36.48
Pimpinella anisum	98.57	99.48	38.21	67.45	12.14	25.98
Ocimum selloi	100.00	100.00	25.36	20.21	6.79	4.99
Baccharis dracunculifolia	92.14	98.43	75.00	18.11	2.86	1.84
Laurus nobilis	98.21	99.48	24.29	13.91	3.21	-16.01
Citrus sinensis	98.21	100.00	29.64	-2.36	0.00	-0.26
Melaleuca alternifolia	95.36	96.85	-3.21	7.61	1.07	47.51
Eucalyptus globulus	86.43	94.23	2.14	-7.61	-1.43	6.56
Chamaecyparis plumosa	45.36	98.95	2.14	6.04	3.21	50.66
Rosmarinus officinalis	32.14	92.65	-2.50	-42.26	2.86	-2.36
Lippia citriodora	18.93	90.03	4.29	0.79	-1.79	-13.91
Corymbia citriodora	18.57	34.38	-2.14	-5.51	-1.43	38.06
Citrus limon	0.36	-35.96	0.36	-35.96	-0.71	-50.13

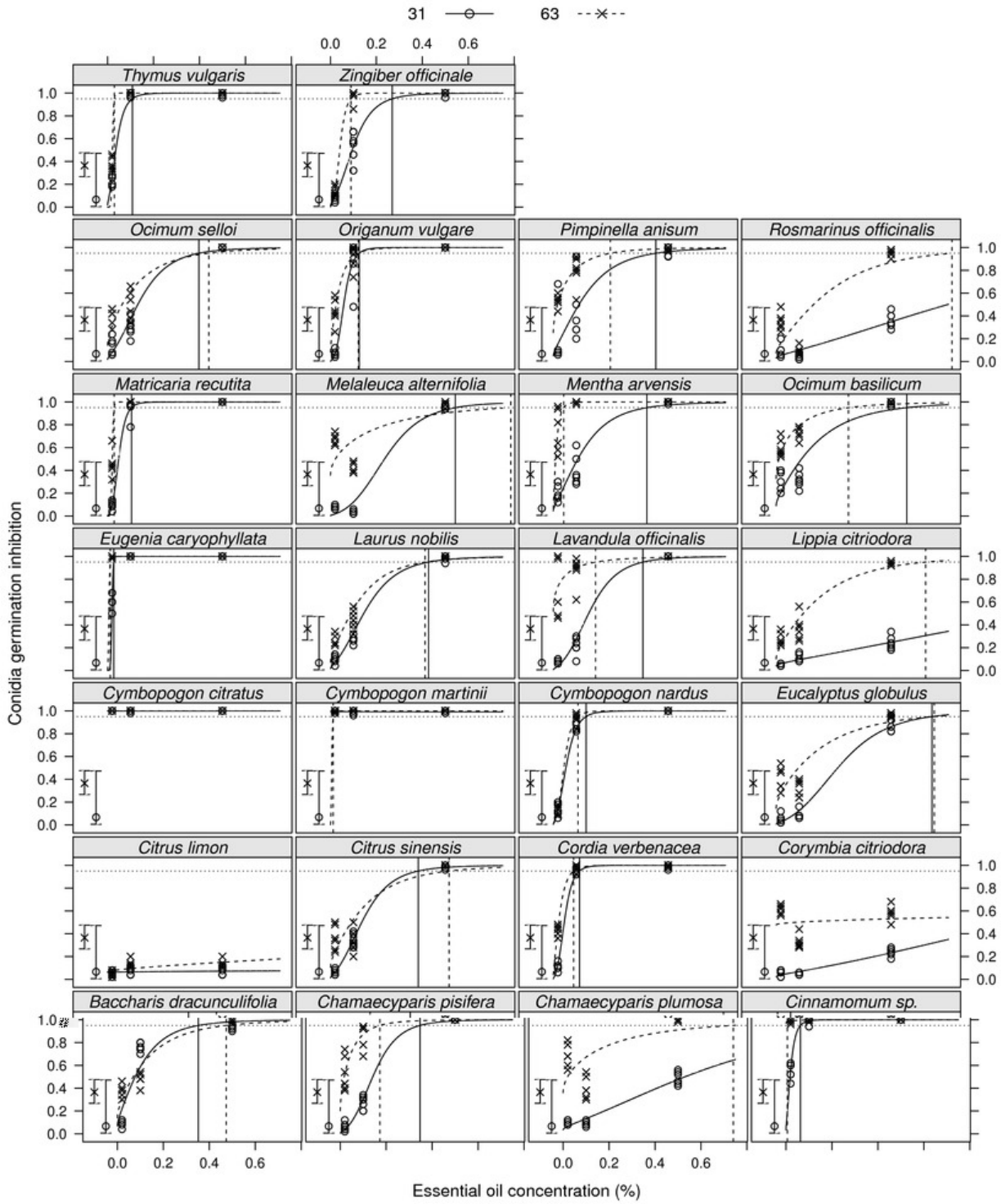

Figure 1 – Regression model fitted to data on number of germinated conidia. The dashed vertical lines represent the concentration that caused 95% of inhibition in strains 63-31 and 63-63 of *Pseudocercospora griseola.* A 95% confidence interval for the proportion of conidial germination in the control is represented along the regression curves. Points represent the proportion of germination observed.

Table 2 – Estimation of coefficients of the logistic regression model for strains 63-31 and 63-63 of *Pseudocercospora griseola* treated with essential oils *in vitro*.

Essential oils	Strain 63-31				Strain 63-63			
	β0	β1	c95%i	p0.5	β0	β1	c95%i	p0.5
Citrus limon	-2.73	0.23	587.39	0.07	-2.69	1.35	17.49	0.07
Corymbia citriodora	-3.74	3.61	3.43	0.23	-0.09	0.30	103.63	0.23
Lippia citriodora	-3.19	2.94	4.36	0.25	-2.18	6.36	0.65	0.25
Rosmarinus officinalis	-3.44	3.98	2.57	0.35	-2.52	6.25	0.76	0.35
Chamaecyparis plumosa	-3.31	4.55	1.89	0.48	-0.57	4.09	0.74	0.48
Eucalyptus globulus	-4.98	9.62	0.68	0.86	-1.77	5.69	0.69	0.86
Melaleuca alternifolia	-5.67	11.68	0.54	0.93	-0.59	3.98	0.79	0.93
Ocimum basilicum	-2.32	6.98	0.57	0.93	-0.71	6.52	0.31	0.93
Pimpinella anisum	-2.95	8.82	0.45	0.96	-1.08	8.07	0.25	0.96
Laurus nobilis	-4.12	10.81	0.43	0.97	-2.57	8.58	0.41	0.97
Mentha arvensis	-3.09	9.46	0.41	0.97	-2.69	26.53	0.05	0.97
Baccharis dracunculifolia	-2.60	9.35	0.35	0.98	-1.83	6.93	0.47	0.98
Citrus sinensis	-4.36	11.82	0.38	0.98	-2.14	7.07	0.52	0.98
Lavandula officinalis	-4.98	12.70	0.39	0.98	0.07	6.70	0.18	0.98
Ocimum selloi	-3.99	11.00	0.40	0.98	-1.79	7.14	0.44	0.98
Chamaecyparis pisifera	-5.21	13.84	0.35	0.99	-1.37	10.39	0.17	0.99
Cymbopogon martini	5.21	-0.51	19.50	0.99	-6.45	85.94	0.01	0.99
Cinnamomum sp.	-3.35	25.14	0.06	1.00	3.51	7.45	0.01	1.00
Cordia verbenacea	-5.41	24.72	0.11	1.00	-3.16	20.57	0.09	1.00
Cymbopogon citratus	6.30	1.42	5.56	1.00	22.63	0.00	*	1.00
Cymbopogon nardus	-4.80	20.52	0.14	1.00	-5.52	25.81	0.11	1.00
Eugenia caryophyllata	-14.09	102.78	0.03	1.00	-6.45	85.94	0.01	1.00
Matricaria recutita	-6.26	28.48	0.10	1.00	-15.81	110.48	0.03	1.00
Origanum vulgare	-6.27	26.02	0.13	1.00	-2.42	15.41	0.12	1.00
Thymus vulgaris	-4.00	21.13	0.11	1.00	-16.25	111.47	0.03	1.00
Zingiber officinale	-4.29	13.95	0.27	1.00	-6.10	30.27	0.09	1.00

**203654410150349486686208.00*.

c95%i represents the concentration of essential oils that inhibited 95% of conidial germination. c95%i was calculated as $p = \exp(\beta_0 + \beta_1.\sqrt{c}) / 1 + \exp(\beta_0 + \beta_1.\sqrt{c})$.

p 0.5 is a percentage of germination inhibition estimated for doses of 0.5% (5000 µL.L^{-1}).

Effect of essential oils on conidial ultrastructure

Cymbopogon martini, *C. citratus* and *E. caryophyllata* were selected for further tests, in function of their potential for inhibiting germination of the pathogen's conidia at concentrations of 0.02%, 0.1% and 0.5%, as observed in this experiment. When both *P. griseola* strains studied here (63-31 (Figure 2A) and 63-63 (Figure 3A)) were treated with sterile water (control) the conidia kept the integrity of the cell wall, the plasma membrane, organized cytoplasm and some visible organelles, such as nucleus and mitochondria, showing well-defined envelopes. In contrast, conidia of both strains that were exposed to essential oils at 0.1% showed clear ultrastructural changes, which were observed by TEM.

The treatment of *P. griseola* conidia with *C. martini* essential oil revealed an altered cytoplasm, accompanied by a large empty area produced by previous cytoplasmic leakage (Figures 2B, 2D, 3B and 3C). Vacuolisation was also frequently noted (Figures 2C and 3C) and electron-dense aggregates covering parts of the wall and of the condensed cytoplasm (Figures 2C and 2D) respectively.

Breakage of the plasma membrane and wall was caused in this conidium (Figure 3C). The accumulation of electron-dense material characterized by an electron dark image, due to the contact with osmium, can indicate penetration by the essential oil. Some mitochondria underwent extensive disruption of the internal structure, with a decrease in the mitochondrial cristae (Figures 2B, 2C, 3B, 3C).

In conidia treated with *C. citratus* essential oil, increased cytoplasmic condensation and aggregation was noted (Figures 2E and 3D). It was also possible to see massive vacuolation of the cytoplasm with vacuole fusion, disruption of the internal mitochondrial structure (Figures 2E, 2F and 3D), large empty spaces caused by absence of the cytoplasmic matrix and lysis of membranous organelles (Figures 2E, 2F, 2G, 3D and 3E) and electron-dense compounds over the aggregated cytoplasm, mitochondria and wall (Figures 2E, 2F and 3D). Rupture of the plasma membrane and wall was observed (Figure 3E), and the wall appeared thinner than normal. The cytoplasm in this conidium was quite disorganized.

Table 3 – Chemical composition of essential oils with antifungal activity of more than 90% on germination of strains 63-31 and 63-63 of *Pseudocercospora griseola*, when tested at 0.1%.

Essential oils / Contents	*Thymus vulgaris*	*Cordia verbenacea*	*Cymbopogon citratus*	*Eugenia caryophyllata*	*Matricaria recutita*	*Cymbopogon martini*	*Cinnamomum* sp.
benzaldehyde	-	-	-	-	-	-	0.28
α-bisabolol oxide A	-	-	-	-	17.77	-	-
α-bisabolol oxide B	-	-	-	-	10.43	-	-
bornyl acetate	-	-	-	-	-	-	0.93
camphene	1.62	-	-	-	-	-	-
chamazulene	-	-	-	-	9.29	-	-
cinnamaldehyde	-	-	-	-	-	-	92.36
cinnamyl acetate	-	-	-	-	-	-	1.48
α-copaene	-	-	-	-	-	-	0.29
1,8-cineole	-	-	-	-	-	-	1.05
eugenol	-	-	-	91.94	-	-	-
trans-β-farnesene	-	-	-	-	31.17	-	-
trans,*trans*-α-farnesene	-	-	-	-	2.18	-	-
geranial	-	-	58.89	-	-	13.72	-
geraniol	-	-	2.61	-	-	75.92	-
α-humulene	-	-	-	1.51	-	-	-
hydrocinnamaldehyde	-	-	-	-	-	-	0.66
isoborneol	3.45	-	-	-	-	-	-
limonene	-	-	-	-	-	-	0.29
menthone	-	14.17	-	-	-	-	-
neral	-	-	38.50	-	-	5.71	-
neryl acetate	-	-	-	-	-	4.65	-
α- pinene	-	-	-	-	-	-	0.54
β- pinene	-	-	-	-	-	-	0.10
pulegone	-	68.96	-	-	-	-	-
sabinene	0.95	-	-	-	-	-	-
terpinen-4-ol	-	-	-	-	-	-	0.28
α-terpineol	-	-	-	-	-	-	1.04
thymol	33.72	-	-	-	-	-	-
not identified	60.26	16.87	-	6.55	29.16	-	0.70

Content of constituents was determined from a peak relative to the total area in GCMS analysis.

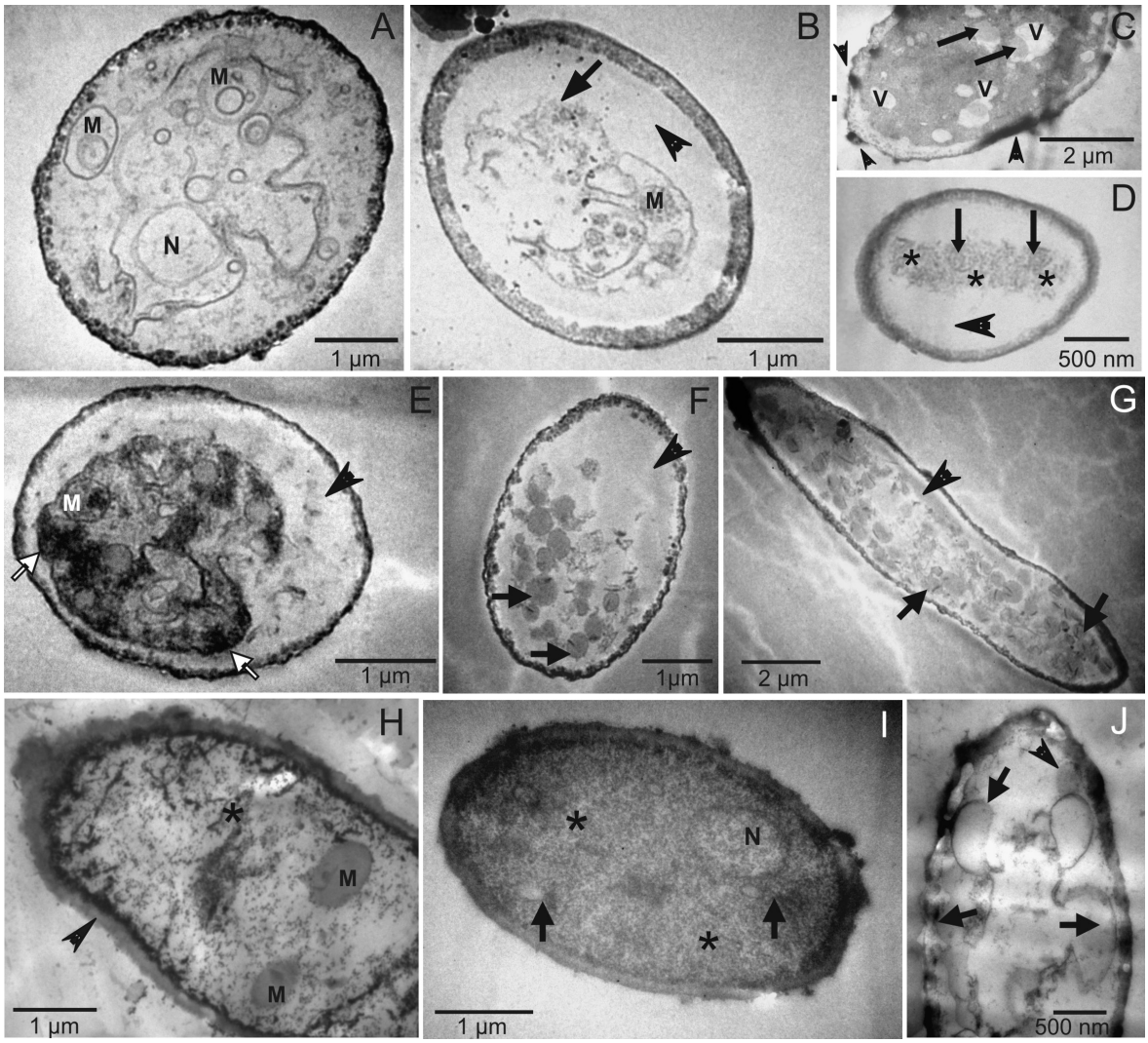

Figure 2 – Transmission electron micrographs of effect of essential oils (EO) at 0.1% on ultrastructure of *Pseudocercospora griseola* conidia, strain 63-31. A, Control, mitochondria (M), nucleus (N). B to D, treatment with *Cymbopogon martini* EO. B, the degraded cytoplasm (arrow), a large empty area (arrowhead) and mitocondria (M) are shown. C, condensation of cytoplasm, some vacuoles (V), mitochondria (arrows) and electron-dense material over the wall (arrowheads) are observed. D, electron-dense aggregates (asterisks) and large empty space are visible (arrowhead), and some organelles are not recognizable (arrows). E to G, treatment with *Cymbopogon citratus* EO. E, condensation of cytoplasm and electron-dense material (arrows), empty area (arrowhead), mitochondria (M) were visible. F and G, a large empty space (arrowheads) and numerous mitochondria with narrow bands of electron-dense compounds (arrows) are shown. H to J, treatment with *Eugenia caryophyllata* EO. H, the area in the wall appears with higher contrast (arrowhead), electron-dense aggregates (asterisk) and mitochondria (M) are observed. I, the nucleus is still visible (N) and other organelles are not well distinguished (arrows). Electron-dense aggregates (asterisks) appeared covering all conidia. J, plasmalema retraction (arrows), mitochondria-like organelle (arrowhead), electron-dense material over the wall are observed in this conidium.

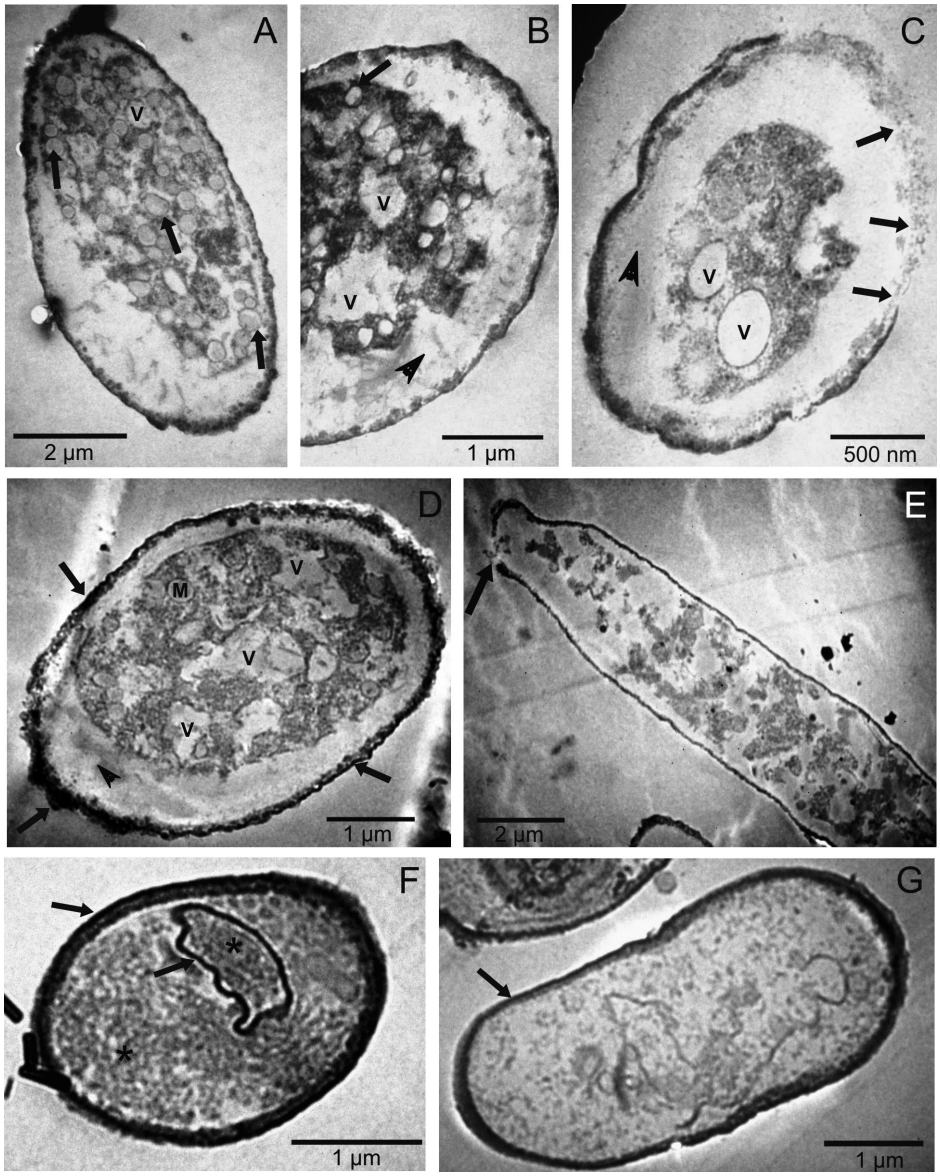

Figure 3 – Transmission electron micrographs of effect of essential oils (EO) at 0.1% on ultrastructure of *Pseudocercospora griseola* conidia, strain 63-63. A, Control, showing numerous mitochondria (arrows), vacuole (V). B and C, treatment with *Cymbopogon martini* EO. B, the cytoplasm is still visible and condensed but most organelles are affected as mitochondria (M); some vacuoles (V) are at times observed within electron-dense material. A large empty area (arrowhead) is shown. C, this conidium is altered; the organelles are difficult to distinguish. Some vacuoles (V), plasma membrane breakage (arrows) and (arrowhead) a large empty area is observed. D and E, treatment with *Cymbopogon citratus* EO. D, in this conidium the condensed cytoplasm, mitochondria (M), vacuoles (V), empty area (arrowhead) and electron-dense compounds (arrows) over the wall are visible. E, in this probably unviable conidium the organelles are not recognizable and the plasma membrane is broken (arrow). F and G, treatment with *Eugenia caryophyllata* EO. F, electron-dense material over the wall and envelope of organelle, and what appears to be the nucleus is observed (arrows). Electron-dense aggregates (asterisks) appeared covering all conidia. G, electron-dense compounds over the wall and remains of organelles and/or cytoplasmic material are shown.

E. caryophyllata essential oil caused disorganization of the cytoplasm, plasmalema retraction, undulation, and invagination in the treated conidia (Figure 2J). Electron-dense material appeared covering all conidia (Figures 2I and 3F), and over the wall (Figure 3F).

It should be emphasized that most of this damage is irreversible, and the changes showed general disorganization of the cytoplasm, as well as cytoplasm leakage, probably caused by loss of conidial membrane integrity, which could eventually lead to fungal cell death. These studies allow us to observe the complexity of the composition of essential oils, and to suggest that this complexity implies that there are multiple mechanisms of action. The mode of action of essential oils commonly affects several targets at the same time, and no particular resistance or adaptation to essential oils has been described (CARSON et al., 2002; BAKKALI et al., 2008). In this regard, Denyer (1990) affirms that leakage of intracellular material is a general phenomenon provoked by many antimicrobial substances. Cell death may have been the result of the extensive loss of cell contents, the exit of critical molecules and ions, and the initiation of autolysis. Besides this, the permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (ARMSTRONG, 2006). It may be that chain reactions from the wall or the outer cell membrane invade the whole cell, through the membranes of different organelles, such as mitochondria and peroxisomes (BAKKAKLI et al., 2008). In this study this membrane permeability was confirmed by observations made under transmission electron microscopy and by the high germination inhibition seen in treated conidia.

The antifungal activity of at least 93% in conidia treated with *Cinnamomum* sp., *E. caryophyllata*, *M. recutita, C. verbenacea*, *T. vulgaris*, *C. citratus* and *C. martini* at 0.1% corroborates similar studies carried out in several other pathosystems. In these, inhibition of germination or mycelial growth caused by pure essential oils, or by one or more of their constituents identified as major in this study (Table 3), could be noted on applying different concentrations and in various forms (WILSON et al., 1997; KASALI et al., 2001; SAIKIA et al., 2001; PARANAGAMA et al., 2003; KISHORE et al., 2007; DE OLIVEIRA et al., 2010; BASSOLÉ et al., 2011; KHAN; AHMAD, 2011). Control of *P. griseola* using essential oils, however, is reported here for the first time.

Other important observations made in this work, were a discontinuous and highly undulating plasma membrane, and extensive disruption of the internal mitochondrial structure with a decrease in the cristae, which can indicate severely altered membranes in both strains of *P. griseola* exposed to essential oils. According to detailed research into membrane permeability (SIKKEMA et al., 1995; VERCESI et al., 1997), some chemical constituents of essential oils, such as typical lipophiles, can pass through the cytoplasmic wall and membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids and permeabilize them. Cytotoxicity appears to include such membrane damage. In eukaryotic cells, essential oils can provoke depolarisation of the mitochondrial membranes by decreasing the membrane potential, affecting Ca2+ ion cycling.

Transmission electron microscopy was seen to be a useful tool to elucidate the effects of *C. martini, C. citratus* and *E. caryophyllata* essential oil at 0.1% on conidia of *P. griseola* strains. It was observed that changes that occurred in the ultrastructure of this pathogen corroborate findings by several researchers in other pathosystems, as reported in (ROZWALKA et al., 2010; RASOOLI et al. 2006; TIAN et al., 2012; AVIS et al., 2009). Scanning and transmission electron microscopy examinations reveal ultrastructural alterations in several compartments of the cell, such as plasma membrane, cytoplasm (swelling, shrivelling, vacuolations, leakage) and nucleus (SOYLU et al., 2006; SANTORO et al., 2007; TYAGI; MALIK, 2010, DAN et al., 2010).

The determination of the biological activity of secondary metabolites from medicinal plants, with respect to direct antimicrobial activity or by activating defense mechanisms of the treated plants, as well as fractionation and identification of these metabolites, may help researchers gain more knowledge to improve their use as an alternative method for plant disease control (STANGARLIN et al., 1999). According to Riefler et al. (2009), considering the multiple biological activities of essential oils and of their chemical constituents, further research involving product formulations and effective blends and doses of isolated compounds should be performed, with the aim of protecting plants efficiently without exerting phytotoxic side-effects.

CONCLUSIONS

Fungitoxic potential is related to the chemical composition of the essential oil, as well as to the sensitivity of the pathogen to one or more constituents in different amounts.

The identification of secondary metabolites in the essential oils with antifungal activity constitutes a useful tool for the synthesis of new products by the chemical industry.

The direct fungitoxic action of several essential oils caused damage to cellular ultrastructure, consequently invalidating the germination of *P. griseola*; it presents a promising alternative for the control of angular leaf spot in bean and represents less risk to human health and the environment.

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