Antifungal activity of essential oils in Colletotrichum lindemuthianum and alternative control of bean anthracnose

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Research Article

Keywords: Phaseolus vulgaris, Defense enzymes, Transmission electron microscopy, Alternative control of plant diseases

Posted Date: August 13th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4705402/v1

Abstract

Anthracnose, caused by *Colletotrichum lindemuthianum*, is the major disease of the common bean and causes serious economic losses. In the present study, we evaluated: (i) the antifungal activity of 26 essential oils (EOs) in the germination and mycelial growth of pathogen races 65 and 73, (ii) ultra structural changes of *Cymbopogon citratus*, *Eugenia caryophyllata* and *Cymbopogon martini* EOs in fungal conidia, (iii) the effect of these EOs on anthracnose control, applied before and after inoculation, and (iv) the effect of *C. citratus* EOs on the activity of defense enzymes on bean plants. Among the 26 EOs, *C. citratus*, *C. martini*, *Cinnamomum* sp., *T. vulgaris*, *E. caryophyllata* and *M. recutita* inhibited 95% conidial germination of fungal strains in concentrations lower than 0.1%. Mycelial growth was completely inhibited by *C. citratus*, *E. caryophyllata* and *C. martini* at 0.1% for 65 and 73 races. There was little mycelial growth in the fungicide treatment 4 DAI. Transmission electron microscopy (TEM) ultrastructure showed that *C. citratus*, *C. martini* and *E. caryophyllata* had direct antifungal action against *C. lindemuthianum*. These EOs caused serious ultrastructural damage to conidia, such as vacuolization, cytoplasm leakage and invagination of plasma membrane. *Cymbopogon citratus* and *E. caryophyllata* reduced anthracnose severity in both preventive and curative experiments. The activity of plant defense enzymes increased in the treatment with *C. citratus*. Essential oils show potential for the management of bean crop disease, especially anthracnose caused by *C. lindemuthianum*.

Introduction

Common bean (*Phaseolus vulgaris* L.) is considered one of the most important grain legumes worldwide for direct human consumption (FAO 2024). In 2022, Brazil produced 2.84 million tons of beans in 2.607.616 ha harvested (Faostat 2024). However, anthracnose caused by the hemibiotrophic fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) (Briosi & Cavara, 1889) is the major disease of common bean and cause significant yield loss, affecting bean crops in Latin America and Africa (Padder et al., 2017).

Disease control of *C. lindemuthianum* is not easy, mainly due to its transmission through contaminated seeds and the high pathogenic variability (Gadaga et al., 2020; Padder et al., 2017). In Brazil, races 65 and 73 are the most frequently observed in bean crops (Pinto et al., 2012). Currently, chemical control and resistant cultivars are the main methods for controlling anthracnose on beans. The use of fungicides can favor the selection of resistant plant pathogens and cause adverse effects on human health and the environment (Fisher 2018). However, new disease control alternatives may replace fungicides and contribute to modern and more sustainable agriculture to protect plants and maintain a less pesticide-dependent disease defense system (Ballaré, 2014). Natural pesticides such as essential oils (EOs) can have a dual effect, acting directly against the pathogen and indirectly on the plant, thus inducing defense responses to the pathogen. Induced resistance is an important alternative control that triggers dormant defense mechanisms in response to both biotic and abiotic elicitors such as EOs, offering new possibilities for practical use in protecting crops against diseases (Jamiołkowska 2020; Stenger et al., 2021).

EOs of medicinal and aromatic plants have the property of inhibiting microbial and fungal development (Sarto, 2014; Wang et al., 2019; Álvarez-Martínez et al., 2021). In addition, oil compounds are eco-friendly, biodegradable and have low toxicity to mammals, which makes them of great potential for use in integrated pest management (Zaker, 2016). EOs have been reported to be effective against some pathogenic fungi. Oil from *Thymus* spp. has been shown strong antifungal activities against *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Phomopsis*, *Trichoderma*, *Trichophyton*, *Microsporon*, and *Epidermophyton* spp. (Soković et al., 2009). Oregano and thyme oils displayed to strongly inhibit the mycelial growth of *C. lindemuthianum*, *Didymella rabiei*, *F. solani*, *Botrytis cinerea*, *Alternaria* spp., *Phytophthora infestans*, *Verticillium fungicola*, and more (Daferera et al., 2003; Lopez-Reyes et al., 2013; Pane et al., 2013; Regnier et al., 2014; Soković & van Griensven, 2006; Soylu et al., 2006). Lemongrass and basil oils were reported to exhibit significant protection against *P. capsici*, *P. drechsleri*, and *P. melonis* (Amini et al., 2016). Ultra structural studies of the effects of EOs on phytopathogenic fungi have also been reported (Perina et al., 2014; Wu et al., 2023). EOs may damage the fungal membrane and promote electrolyte leakage, resulting in fungal death. Additionally, a high oil concentration *in vitro* may cause ultra structural changes such as nucleolar segregation, lipid degeneration and damaged mitochondria) (André et al., 2017). Therefore, EOs may act either as fungitoxic substances (Wang et al., 2019) or by inducing plant resistance (Jamiołkowska, 2020; Stenger et al., 2021).

There are few reports in the literature on the antifungal activity and mode of action of EOs in *C. lindemuthianum*, with a view to using these oils as an environmentally friendly and economical alternative in integrated disease management. The objectives of this study were to (i) assess antifungal activity of 26 EOs on the germination and mycelial growth of pathogen races 65 and 73, (ii) evaluate ultrastructural changes of oils *Cymbopogon citratus*, *Eugenia caryophyllata* and *Cymbopogon martini* in fungal conidia, (iii) study the effect of oils on controlling anthracnose, applied before and after inoculation, and (iv) evaluate the effect of *C. citratus* oil on the activity of defense enzymes on bean plants.

Material and methods

Fungal isolates

Two single-spore isolates of *C. lindemuthianum*, one of race 65 (LV 136) and the other of race 73 (LV 99) were used in this present study. These isolates were deposited in the collection of the Laboratory of Plant Resistance to Diseases, Department of Biology, Federal University of Lavras, Brazil. The isolates were maintained in culture medium M3 (Junqueira et al., 1984) and incubated in a growth chamber at 22 ± 2°C under a 12h photoperiod for 15–20 days.

Essential oils

EOs extracted from 26 medicinal, aromatic, or forestry plants of 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* ChaixexVill.,*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 alternifolia* Cheel), Poaceae (*Cymbopogon citratus* DC) Stapf., *Cymbopogon martini* (Roxb.)., *Cymbopogon nardus* (L.) Rendle), Rutaceae (*Citrus limon* (L.) Burm.f., *Citrus sinensis* (L.) Osbeck.), Verbenaceae (*Lippia citriodora* Kunth), and Zingiberaceae (*Zingiber officinale* Roscoe) were used in this study.

Eos of *Lavandula*, *Ocimum*, *Origanum*, and *Cinnamomum* were extracted by hydrodistillation in a modified Clevenger-type apparatus. The first three plants were collected in the Garden of Medicinal Plants of the Federal University of Lavras, Minas Gerais, Brazil. *Cinnamomum* barks were bought at local store. The other oils were supplied by Chamel Natural Products Industry and Trade Ltd., Paraná, Brazil. All oils were kept in a refrigerator at 4°C and protected from light in amber vials.

Effect of EOs on conidial germination of *C. lindemuthianum*

All 26 oils were diluted in sterile distilled water containing Tween 20, 0.1%; the final concentrations were 0.02, 0.1, and 0.5%. Forty µL aliquots of diluted oils and 40µL of conidial suspension of *C. lindemuthianum* 1.2 x 10^6 conidia/mL (concentration in all experiments) were placed in polypropylene 96-well plates. Control treatment received 40 µL of conidial suspension and 40 µL of sterile distilled water.

Germination was halted after 12h incubation at 22 ± 2ºC with 20 µL lactoglycerol cotton blue. For each treatment, fifty conidia for each repetition were evaluated under a stereo microscope. The conidia were considered to have germinated when the conidia germ tube length was longer than their diameter. The experimental design was completely randomized. Six replicates were prepared for each treatment. The effect of EOs on conidial germination was assessed using the factorial arrangements 26 x 3 x 2 (oil x concentrations x races) and a control treatment. Each plate well was an experimental unit.

Data analysis was performed by generalized linear models assuming a logit link and a binomial distribution. The linear predictor is represented by $\log [p_i / (1- p_i)] = \beta_{0i} + \beta_{1i}. \sqrt{c}$, where p_i = probability of inhibition of fungal germination when using the EO i (i = 1, 2,..., 26) at concentration c (c = 0.02, 0.1 and 0.5%). β_{0i} and β_{1i} are parameters that shape the relationship. Inference was conducted by considering Pearson residuals' estimated over dispersion parameter (φ).

Effect of EOs on mycelial growth of *C. lindemuthianum*

Before performing the experiment, we measured pH of each treatment [PDA medium (42 g/L potato-dextrose-agar, KASVI, India) plus products]: with EOs of *C. citratus* pH 5.7; *E. caryophyllata* pH 5.68; *C. martini* pH 5.68; Azoxystrobin fungicide (250 g/L a.i., Syngenta, Basel, Switzerland) 5.65; ASM (acibenzolar-S-methyl) pH 5.36; sterile distilled water + Tween 20, 0.1% pH 5.66; sterile distilled water pH 5.63.

Each race of *C. lindemuthianum* received 0.1% oil of *C. citratus*, *E. caryophyllata*, and *C. martini* since oils inhibited 95% germination at this concentration or less. The test was performed in Petri dishes 9 cm in diameter with PDA medium. The oils were added to the medium before being poured on Petri dishes after the temperature fell to 50°C, so the final concentration reached the levels determined by the trial. The control treatments comprised sterile distilled water and sterile distilled water plus Tween 20, 0.1%. Azoxystrobin fungicide at 0.4 g L^{-1} was used as an inhibition pattern, and Bion (acibenzolar-S-methyl, ASM) at 0.08 g L^{-1} was used as the standard induced resistance in seven treatments.

Fungal plugs of 5 mm containing young mycelia were placed in the center of each plate, which was incubated at 22 ± 2°C and 12 h photoperiod until the end of the evaluation. The experimental design was completely randomized. Eight replicates were prepared for each treatment, and each plot consisted of a plate. The diameter of colonies was evaluated every two days from inoculation until control mycelium occupied the whole medium surface. Using these data, we calculated the mycelial growth rate (MGR) according to the formula proposed by Maguire (1962).

Effect of EOs on the conidial ultra structure of *C. lindemuthianum*

This study was conducted only with race 65 of *C. lindemuthianum*, which has greater geographical spread and high virulence in Brazil (Balardin & Kelly, 1997; Pinto et al., 2012). The oils were *C. citratus, E. caryophyllata* and *C. martini* 0.1%. Azoxystrobin fungicide 0.4 g L^{-1} and sterile distilled water with Tween 20, 0.1% were used as control treatments.

Conidial masses were fixed in a modified Karnovsky solution (glutaraldehyde 2.5%, formaldehyde 2.5% in sodium cacodylate buffer 0.05 M pH 7.2, and calcium chloride ($CaCl_2$) 0.001 M). The material was prepared for transmission electron microscopy (TEM) as described by Rozwalka et al., (2010). Each treatment was replicated twice. Sample observation and image recording were performed in a transmission electron microscope Zeiss EM 109, 80 kV.

Chemical composition of EOs

EOs showing antifungal activity higher than 97% at 0.1%, *C. martini* (0.144 mg), *M. chamomilla* (0.127 mg), *E. caryophyllata* (0.200 mg), *T. vulgaris* (0.147 mg), *C. verbenaceae* (0.179 mg), *C. citratus* (0.160 mg) and *Cinnamomum* sp. (0.118 mg) were dissolved in 0.5 mL dichloromethane. One microliter of diluted EOs was injected into a gas chromatograph coupled to a mass spectrometer (GCMS-QP2010 Plus, Shimadzu). The chromatograph used ultrapure helium as carrier gas at a flow rate of 1.8 ml min^{-1}. Separation was performed on a capillary column Equity-5 (30 m x 0.22 mm ID x 0.25 µm) with column oven at 60°C for 2 min, heating at 3°C min^{-1} to 240°C and remaining at 240°C for 15 min. The injector temperature was 220°C, with a manual split injection at 1:15 and a running time of 77 min. The mass spectrometer used interface GC-MS at 250°C, with an ion source at 200°C and an impact ionization mode at 70 eV. Oil constituents were identified by testing the mass spectral similarity between the relative area (%) and GCMS library data (Wiley 8).

Effect of EOs on bean anthracnose control

Two experiments, one preventive and one curative, were conducted in a greenhouse to verify the effect of EOs on anthracnose control. In the preventive experiment, plants of the bean cultivar Perola were grown in a 3 L plastic pot containing a 2:1:1 ratio of Plantmax, soil and sand. The plants were grown in a greenhouse under natural conditions (25 ± 5ºC) over the experimental period. In the V3 stage, plants were hand-sprayed with 3 ml EO of *C. citratus*, *E. caryophyllata*, and *C. martini* 0.1% in distilled water with Tween 20, 0.1%; ASM 0.08 g L^{-1}; azoxystrobin 0.4 g L^{-1} and distilled water up to the point of runoff. At 7 days after spraying (DAS), the plants were spray-inoculated with a conidial suspension of *C. lindemuthianum* and placed in a growth chamber for 48 hours.

The experimental design was randomized complete block. Four replicates were prepared for each treatment, and each experimental plot consisted of one pot containing three plants. Anthracnose severity assessment was carried out on four consecutive dates at weekly intervals starting at 5 DAI. The diagrammatic scale proposed by Godoy et al., (1997) was used. The disease severity values from the 4 anthracnose assessments were integrated as area under the disease progress curve (AUDPC), following the method proposed by Shaner & Finney (1977). When *F* test was significant, means were compared by the Scott Knott test ($p \leq 0.05$).

In the curative experiment, bean plants obtained as in the previous experiment were inoculated at the V3 stage. At 7 DAI, all treatments from the previous experiment were sprayed except for the ASM treatment. The experimental design, anthracnose assessments, AUDPC determination, and data analysis were performed as described for the previous experiments.

Effect of EO of *Cymbopogon citratus* in the activity of defense enzymes

Bean plants were obtained and inoculated as described in the preventive experiment. Treatments consisted of ASM, oil of *C. citratus* and, and one control in plants inoculated with race 65 of *C. lindemuthianum* and non-inoculated plants. For enzyme analysis, leaf samples were collected at 3, 4, and 5 DAS. The inoculation occurred at 3 DAS. After each collection, leaves were frozen in liquid nitrogen and stored at -80^{o}C until maceration.

One gram of each powder sample was placed in tubes. Five mL of potassium phosphate buffer 100 mM pH 7.0 was added per sample and tubes were homogenized for 10 seconds. The suspension was centrifuged at 12.000 rpm for 15 minutes (4°C), and the supernatant was used as an enzyme source.

Enzymatic activity

The total protein of the enzyme extract was determined using the Bradford method (1976), using the bovine serum albumin (BSA) standard curve. Chitinase (CHI; EC 3.2.1.14) activity was evaluated according to Wirth & Wolf (1990), using substrate CM-Chitin-RBV. CHI activity was expressed in activity units per milligram of protein per minute. Polyphenol oxidase (PPO; EC 1.10.3.1) activity was measured

by converting catechol to quinone (Gauillard et al., 1993), using the molar extinction coefficient 3,450 $M^{-1}cm^{-1}$ and expressed by quinone formation per milligram of protein per minute. Guaiacol peroxidase (POX; EC 1.11.1.7) activity was calculated according to the method by Urbanek et al. (1991), using the molar extinction coefficient 26.6 $M^{-1}cm^{-1}$ and expressed in µmol of oxidized H_2O_2 per milligram of protein per minute. Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity was measured according to Mori et al. (2001). The PAL activity values were expressed in µmol of trans-cinnamic acid per milligram of protein per minute using the molar extinction coefficient 30.5 $M^{-1}cm^{-1}$. The experimental design was randomized complete block. Four replicates were prepared for each treatment, and experimental plots comprised four plants per collection. Enzymatic activities were compared using the Duncan test at 5% probability at each collection time.

Results

Effect of EOs on conidial germination of *C. lindemuthianum*

Analysis of deviance results showed a significant interaction, indicating that differences occurred among treatments for germination percentage. All 26 oils at 0.5% inhibited conidial germination 87–100% in pathogen race 65, whereas 17 oils showed these results in race 73. The oils of *C. citratus, C. martini, Cinnamomum sp., T. vulgaris, E. caryophyllata*, and *M. recutita* inhibited 95% germination in both races of *C. lindemuthianum* at the lowest concentrations, 0.01–0.07% (Table 1 and Fig. 1).

Table 1
Estimation of coefficients of logistic regression model for races 65 and 73 of *Colletotrichum lindemuthianum* treated with essential oils *in vitro*.

β0		β1	c95%i	p0.5	β0	β1	c95%i	p0.5
Melaleuca alternifolia	-1,31	4,52	0,89	0,87	-0,97	0,44	78,62	0,34
Chamaecyparis pisifera	-1,15	6,65	0,38	0,97	-0,36	0,15	457,63	0,44
Pimpinella anisum	-1,23	8,40	0,25	0,99	-0,03	0,60	24,54	0,60
Ocimum selloi	-1,84	7,16	0,45	0,96	-0,65	1,57	5,22	0,61
Eucalyptus globulus	-4,96	31,51	0,06	1,00	-1,06	2,17	3,40	0,62
Corymbia citriodora	-2,34	9,32	0,32	0,99	-1,19	2,48	2,78	0,64
Citrus limon	-2,14	13,04	0,15	1,00	-0,04	1,00	8,85	0,66
Lippia citriodora	0,79	5,86	0,14	0,99	-1,09	3,18	1,61	0,76
Mentha arvensis	-1,60	8,60	0,28	0,99	-1,63	3,98	1,32	0,77
Ocimum basilicum	-1,43	8,16	0,29	0,99	-0,73	3,81	0,93	0,88
Baccharis	-1,91	8,91	0,30	0,99	-1,79	5,65	0,70	0,90
Rosmarinus officinalis	-0,97	5,86	0,45	0,96	-2,70	7,63	0,55	0,94
Lavandula officinalis	-2,48	8,59	0,40	0,97	-2,02	7,14	0,48	0,95
Cymbopogon nardus	-1,84	6,94	0,48	0,96	-1,81	7,05	0,45	0,96
Chamaecyparis	-1,25	6,82	0,38	0,97	-1,17	6,24	0,43	0,96
Origanum vulgare	-	117,15	0,03	1,00	-1,60	6,58	0,48	0,96
Cordia verbenacea	-0,94	6,89	0,32	0,98	-0,96	6,23	0,39	0,97
Laurus nobilis	-	115,83	0,03	1,00	-1,35	9,00	0,23	0,99
Zingiber officinale	-1,91	7,21	0,45	0,96	-	117,39	0,03	1,00
Citrus sinensis	-5,20	34,46	0,06	1,00	-2,43	11,53	0,22	1,00
** 18350045018960200000000.00*								
c95%i representa a concentração do óleo essencial que inibe 95% da germinação dos conídios, foi calculado como $p = \exp(\beta_0 + \beta_1.\sqrt{c}) / 1 + \exp(\beta_0 + \beta_1.\sqrt{c})$.								
p 0,5 é a porcentagem de inibição da germinação estimada para a concentração de 0,5%.								

β0		β1	c95%i	p0.5	β0	β1	c95%i	p0.5
Matricaria recutita	-2,82	21,11	0,07	1,00	-4,32	27,26	0,07	1,00
Eugenia caryophyllata	-	121,23	0,03	1,00	-4,68	29,33	0,07	1,00
Thymus vulgaris	-	113,49	0,03	1,00	-2,06	20,80	0,06	1,00
Cinnamomum sp.	-	116,48	0,03	1,00	-	117,23	0,03	1,00
Cymbopogon martini	-	106,49	0,02	1,00	-2,93	27,30	0,05	1,00
Cymbopogon citratus	23,63	0,00	*	1,00	-7,26	91,66	0,01	1,00
** 1835004501896020000000.00*								
c95%i representa a concentração do óleo essencial que inibe 95% da germinação dos conídios, foi calculado como $p = \exp(\beta_0 + \beta_1.\sqrt{c}) / 1 + \exp(\beta_0 + \beta_1.\sqrt{c})$.								
p 0,5 é a porcentagem de inibição da germinação estimada para a concentração de 0,5%.								

According to the estimated logistic regression coefficients, an increase in the concentration of *C. sinensis*, *L. nobilis*, *C. verbenacea*, and *O. vulgare* from 0.1 to 0.22, 0.23, 0.39, and 0.48%, respectively, may inhibit 95% conidial germination in race 73. The increased concentration of most EOs promotes increased antifungal activity against both races of *C. lindemuthianum*, and the conidial germination of race 65 was more inhibited (Table 1).

Effect of EOs on mycelial growth of *C. lindemuthianum*

The treatment showing the highest mycelial growth rate in both races was sterile distilled water + Tween, followed by the treatment with sterile distilled water alone, without statistical difference. The ASM treatment differed from the two others in that it seemed to slightly inhibit fungal growth. The other treatments had no growth. The oils of *C. citratus*, *E. caryophyllata*, and *C. martini* 0.1% completely inhibited mycelial growth in both races. Fungicide treatment showed little growth after 4 days and was not statistically different from the EOs (Fig. 2).

Effect of EOs on the conidial ultra structure of *C. lindemuthianum*

The EOs of *C. citratus*, *E. caryophyllata*, and *C. martini* were selected for their potential to inhibit conidial germination in *C. lindemuthianum* at 0.02, 0.1, and 0.5%. When conidia of race 65 of *C. lindemuthianum* were treated with sterile distilled water (control), the treatment did not affect the integrity of the cell wall, plasmalemma, or cytoplasm; some organelles such as nucleus and mitochondria showed well-defined envelopes. In contrast, conidia treated with fungicide and oils at 0.1% exhibited ultra structural changes (Fig. 3A).

The treatment of conidia with fungicide caused cytoplasmic vacuolization, invagination of the plasmalemma, and disruption of the mitochondrial internal structure with decreased cristae (Fig. 3B). Accumulation of electro-dense material covered portions of the cytoplasm, which was condensed due to contact with osmium. This may indicate penetration of a fungitoxic agent, in this case of synthetic origin (Fig. 3B and 3C). Also, some conidia were very degenerate and had rupture of the plasmalemma (Fig. 3C).

Conidia treated with the EO of *C. citratus* exhibited significant changes, including intense vacuolization and condensation of the cytoplasm (Fig. 3D). The accumulation of electro-dense clusters covering parts of the condensed cytoplasm indicated the penetration of a fungitoxic agent of biotic origin (Fig. 3D and 3E). Plasmolysis of conidia, large voids produced by previous cytoplasm leakage, and lysis of membranous organelles were observed. Also, the cell wall was thinner (Fig. 3E), and there was electro-dense material (Fig. 3D and 3E).

The EO of *E. caryophyllata* caused vacuolization, fusion of vacuoles, cytoplasm condensation, and disruption of the mitochondrial internal structure with decreased cristae (Figs. 3F and 3G). Cytoplasm leakage and electro-dense compounds covered parts of the aggregated cytoplasm (Fig. 3F and 3G). Conidia exposed to the oil of *C. martini* had voids (Fig. 3H), intense vacuolization, shrinkage, and invagination of the plasmalemma (Fig. 3I), cytoplasmic condensation, electron-dense aggregates and vesicles (Fig. 3H and 3I).

Chemical composition of EOs

The identification and composition of the main chemical constituents of EOs reveal a abroad diversity. Cinnamaldehyde (92.36%) in *Cinnamomum* sp., eugenol (91.94%) in *E. caryophyllata*, geraniol (75.92%) in *C. martini*, pulegone (68.96%) in *C. verbenaceae*, geranial (58.89%) and neral (38.50%) in *C. citratus*, thymol (33, 72%) in *T. vulgaris*, and -b-farnesene (31.17%) in *M. recutita* were found.

Effect of EOs on bean anthracnose control

When assessing the protective effect on bean plants against anthracnose, the treatments using ASM, fungicide and EO of *C. citratus* showed lower disease severity than the control (water). ASM promoted the most significant disease reduction, up to 62%, followed by fungicide and *C. citratus oil*, which reduced 44% and 29%, respectively. These treatments did not show significant differences but differed from ASM and control. Other treatments did not show a significant reduction in the disease severity or differ from the control (Table 2). However, all treatments demonstrated a curative effect by reducing anthracnose severity compared to the control and did not differ by Scott-Knott test ($p < 0.05$). The most significant reduction was caused by the fungicide (28.5%), followed by the EOs of *C. citratus*, *E. caryophyllata*, and *C. martini*, at 21%, 18.7%, and 13.5%, respectively (Table 2).

Table 2
Area under the disease progress curve (AUDPC) and percentage of anthracnose control according to treatments for preventive and curative experiments in greenhouse beans.

	Preventive Experiment		**Curative Experiment**	
Treatments	AUDPC	Control (%)	AUDPC	Control (%)
Water	127c	0	118b	0
C. martini	124c	2	102a	13.5
E. caryophyllata	103c	19	96a	18.7
C. citratus	89b	29	93a	21
Fungicide: Azoxystrobin	71b	44	85a	28.5
ASM: Acibenzolar-S-Methyl	48a	62	-	-
C.V. (%)	21.21	-	9.34	-
Means with same letter in the column do not differ by *Scott-Knott test* ($p < 0.05$).				

The EOs used in these experiments at 0.1% did not produce any phytotoxicity symptoms on beans. Thus, at 7 DAS, the EOs could not have had a proper antimicrobial effect against *C. lindemuthianum*, which had been inoculated seven days after oil application. Instead, oils could have caused induced resistance on beans. Both preventive and curative experiments with *C. citratus* reduced the severity of anthracnose by 29% and 21%, respectively. Also, the treatment did not differ statistically from the fungicide treatment, which reduced disease by 44% and 28.5% in the two experiments, respectively (Table 2).

Effect of EO of *Cymbopogon citratus* in the activity of defense enzymes

The activity of chitinase (CHI) was higher in the ASM treatment at 3 DAS without inoculation with *C. lindemuthianum*. The treatment with the EO of *C. citratus* (Cc) and the control (Te) did not differ; however, they differed from ASM (Fig. 4A). After plant inoculation (after the first collection), at 4 DAS, all treatments had activity similar to CHI. At 5 DAS, the ASM treatment and the treatment inoculated with *C. citratus* oil (Cc) had equal CHI activity, differing from the other treatments (Fig. 4A).

Polyphenol oxidase (PPO) activity at 3 DAS was statistically similar for all treatments. At 4 DAS, PPO activity was higher in plants treated with ASM without *C. lindemuthianum* inoculation. At 5 DAS, which was the last assessment time at 2 DAI, ASM treatment inoculated with *C. lindemuthianum* was the most active, followed by the treatment inoculated with *C. citratus* oil, differing from the other treatments (Fig. 4B). After this period, there could be an increase in enzyme activity when plants recognized the pathogen.

Peroxidase activity (POX) remained similar in all treatments at 3 and 4 DAS. At 5 days, enzyme activity largely increased in plants treated with ASM inoculated with *C. lindemuthianum*, differing from the other treatments (Fig. 5A). Regarding the activity of phenylalanine ammonia-lyase (PAL) 3 to 5 DAS, treatments had no significant effect on both inoculated and non-inoculated plants (Fig. 5B).

Discussion

Natural plant products such as EOs may be promising alternatives to synthetic pesticides and reduce the pesticide effects on human health and the environment. Bean crops face biotic stressors, especially fungal diseases such as anthracnose caused by *C. lindemuthianum*, the major disease of common beans, and cause significant yield loss. The search for effective, affordable, and readily available natural plant products has been recurrent for plant disease management instead of synthetic fungicides, with the development of fungicide resistance (Fisher, 2018; Blazquez, 2014). In this regard, natural products, especially EOs, offer the best alternative for disease management, as they have other advantages. Furthermore, EOs may fit Organic Materials Review Institute (OMRI) standards for certification for pest and plant disease control in crop fields (OMRI, 2024).

Firstly, some of the EOs in this study were effective against *C. lindemuthianum*. For example, *C. citratus*, *C. martini*, *Cinnamomum* sp., *T. vulgaris*, *E. caryophyllata*, *M. recutita*, and *C. sinensis* 0.1% EOs tested possess antifungal activity, being able to inhibit 95% conidial germination of *C. lindemuthianum.* Not surprisingly, the broad efficacy of the EOs occurs because plants with 0.01 to 5% EO content naturally use their oil components to defend themselves against plant diseases (Arshad et al., 2014; Butnariu & Sarac, 2018). Mycelial growth of *C. lindemuthianum* was entirely inhibited by *C. citratus*, *E. caryophyllata*, and *C. martini* 0.1%, which indicates that EOs affect fungi development in their initial stage. Inhibition of germination and/or mycelia growth caused by pure EOs or their constituents may occur in different concentrations and forms of application (Khan & Ahmad, 2011). In previous studies, the volatile geraniol identified as the main constituent of *C. citratus* has been shown to have strong mycelial growth inhibition against *Colletotrichum falcatum* and *Botrytis cinerea* (Singh et al., 2002; Yan et al., 2021).

Secondly, in the current study, the membrane permeability was confirmed by transmission electron microscopy and high germination inhibition in the conidia of *C. lindemuthianum* treated with EOs. Discontinuous wavy plasma membrane and degeneration of inner mitochondrial structure with decreased cristae indicate severely altered membranes in *C. lindemuthianum* conidia exposed to EOs. Based on reports from previous studies, severe damage in conidial ultra structure in two races of *Pseudocercospora griseola* and damage in the plasma membrane cells of *B. cinerea* treated with EOs (Hoyos et al., 2012; Yan et al., 2021). Most of these damages are irreversible, and ultrastructural changes such as disruption and cytoplasmic leakage may be caused by membrane integrity loss, leading to cell death.

The complex composition of EOs suggests multiple mechanisms of action and different invasion targets (Sil et al., 2020). In our study, conidia of *C. lindemuthianum* treated with oil of *C. citratus* showed

plasmolysis, large voids produced by previous cytoplasm leakage, and lysis of membranous organelles. Furthermore, *E. caryophyllata* caused vacuolization, a fusion of vacuoles, cytoplasm condensation, and disruption of the mitochondrial internal structure with decreased cristae, while *C. martini* cause voids, intense vacuolization, shrinkage, and invagination of the plasmalemma, cytoplasmic condensation, electron-dense aggregates and vesicles. It has been suggested that *C. citratus, E. caryophyllata and C. martini* may cause severe ultra structural damage in *C. lindemuthianum* conidia. The membrane damage leads to the leakage of intracellular components to the extracellular matrix (Li et al., 2018). Cell death may occur by great loss of cell contents from important molecules and ions, followed by autolysis. Additionally, permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Yapryntseva, 2024). According to studies of membrane permeability, some compounds of EOs, which are typical lipophilic materials, pass through the wall and cytoplasm membrane (Dwivedy et al., 2016). Such compounds affect the structure of different layers of polysaccharides, fatty acids, and phospholipids, thus permeabilizing them. Cytotoxicity seems to include this damage in the membrane. In eukaryotes, these oils promote depolarization of mitochondrial membranes by lowering the membrane potential, thus affecting the ion cycle of $Ca2^{+}$ (Bakkali et al., 2005). The distortion of cellular structure could cause expansion and disruption of the membrane, thus increasing its passive permeability and fluidity.

Cytotoxic activity of EOs is mainly due to the presence of phenols, aldehydes, and alcohols (Bakkali et al, 2008; Bajpai, 2012). In ascending order of antimicrobial activity of EOs components are phenols, aldehydes, ketones, alcohols, ethers, and hydrocarbons (Sadgrove & Jones 2015). The composition of the main chemical constituents of EOs was determined and revealed a broad diversity. We identified the major components of the three EOs promoting higher germination inhibition: geranial and neral (aldehydes) in *C. citratus*; eugenol (phenol) in *E. caryophyllata* and geraniol (alcohol) in *C. martini*. Previous studies of the antifungal components of EOs reveal that the oils consist of 20–60 components at various concentrations. The main constituents studied here may have a strong antimicrobial effect, which may indicate that these molecules have synergistic antimicrobial activity. The synergistic effects of EOs' major and minor constituents may be taken into account for their antimicrobial activity. Oils could be activating defense mechanisms in plants in the preventive test, since many antimicrobial oil constituents either volatilize quickly or do not stay long in the treated tissues (Bakkali et al, 2008).

Thirdly, concerning the protective effect on bean plants against anthracnose, *C. citratus* and *E. caryophyllata* oils were proven effective and reduced the disease severity by 21% and 18.7%, as shown in the curative experiment and by its high cytotoxicity to *C. lindemuthianum* conidia (Figs. 3D and 3E). Also, *C. citratus* oil had an indirect effect possibly due to induction of defense mechanisms in the plant, as occurred in the preventive experiment. These findings are similar to those reported in other studies. For example, the use of EOs to control Asian soybean rust reduced the severity of disease on soybean plants by between 52% and 80%, even with a single application treated 0, 6, 12, and 24 h before inoculation (preventive treatment) (Da Silva et al., 2012).

Fourthly, concerning the effect of *C. citratus* on the activity of defense enzymes, *C. citratus* and the ASM treatment had equal chitinase activities, at 5 DAS. In contrast, higher chitinase activity was found in plants inoculated with *C. lindemuthianum* 16 days after phosphite treatment, followed by plants treated with ASM (Costa et al., 2017). Our results for PPO activity showed that ASM treatment inoculated with *C. lindemuthianum* was the most active, followed by the treatment inoculated and with *C. citratus*, at 5 DAS. On tomato leaves, an increase in PPO activity induced by *C. citratus* was observed when tomato was inoculated with *Alternaria solani*, at 3 DAS (Hendges et al., 2023). Previous studies have reported that an increase in enzyme activity may cause a high concentration of toxic oxidation products and, thus, a higher level of resistance to pathogens (Zheng-Cuiming et al., 1999).

In our study, all treatments remained similar POX activity at 3 and 4 DAS. Conversely, an increase of peroxidase activity was observed in the second and third pairs of tomato leaves with the treatment of *C. citratus* on tomato inoculated with *Alternaria solani* at 96 h, with a maximum response at 144 h (Hendges et al., 2023). Our findings revealed no significant effect for PAL activity on inoculated and non-inoculated plants from 3 to 5 DAS. Godinez-Vidal et al., (2007) also reported little differences in PAL activity on chilli plants at 7 and 14 DAI with *Nacobbus aberrans*. On biotic and abiotic inducers in *Phaseolus vulgaris* and *Musa* spp. found delayed activity of PAL at 6, 8, and 12 days after treatment (Saravanan et al., 2004).

As observed by Walling (2001), volatiles are potent signals for induced resistance, but must be carefully evaluated. Many constituents of EOs are volatile, have antimicrobial action, and are synthesized immediately after pathogen infection in the plant's defense systems (Brilli, 2019). These volatiles are described as defense signs recognized by membrane receptors that respond by amplifying signals. Such response amplification mechanism generally requires low protein concentrations and can generate more effective and less transient responses than the synthesis of induced resistance enzymes.

EOs are effective against fungal and bacterial pathogens (Sarto, 2014; Wang et al., 2019; Álvarez-Martínez et al., 2021; Parikh, 2021). Oil compounds have low mammalian toxicity, besides being biodegradable, are effective against wild types and pesticide-resistant strains, and economically viable, which makes them of great potential for use in integrated management of plant diseases (Brilli, 2019). In addition, they can be a good alternative for anthracnose control, as they provide direct toxicity to fungi and can also induce defense responses in plants.

In conclusion, the fungitoxic potential is related to both chemical composition of oil and pathogen sensitivity to one or more constituents in different amounts. Direct fungitoxic action of EOs of *C. citratus, E. caryophyllata* and *C. martini* causes severe ultra structural damage in *C. lindemuthianum* conidia, thus invalidating germination. Our data suggests the potential application of three EOs as an alternative control method for pathogens of bean crops, especially *C. lindemuthianum*. Therefore, they can be used in integrated management of anthracnose with less risk to human health and the environment.

Declarations

Acknowledgements The authors wish to thank the Coordination of Improvement of Higher Education Personnel (CAPES) for financial support and doctoral scholarship, the National Council for Scientific and Technological Development (CNPq306133/2021) for productivity grant for the last author, the Foundation for Research Support of Minas Gerais (FAPEMIG) and Studies and Projects Financing Brazilian Agency (FINEP) for financial support to the Laboratory of Electron Microscopy and Ultra structural Analysis of the Federal University of Lavras, Minas Gerais, Brazil.

Conflict of interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data Availability statement: The data that supports the findings of this study are available upon request.

***Research involving human participants and/or animals*:** This study does not include experiments with either human participants or animals.

References

1. Álvarez-Martínez, F. J., Barrajón-Catalán, E., Herranz-López, M., & Micol, V. (2021). Antibacterial plant compounds, extracts and essential oils: An updated review on their effects and putative mechanisms of action. *Phytomedicine*, *90*, 153626. https://doi.org/10.1016/j.phymed.2021.153626.
2. Amini, J., Farhang, V., Javadi, T., & Nazemi, J. (2016). Antifungal Effect of Plant Essential Oils on Controlling Phytophthora Species. *The Plant Pathology Journal*, *32*, 16–24. https://doi.org/10.5423/ppj.oa.05.2015.0091.
3. André, W. P. P., Cavalcante, G. S., Ribeiro, W. L. C., Santos, J. M. L., dos, Macedo, I. T. F., Paula, H. C. B., & de Bevilaqua, C. M. L. (2017). Anthelmintic effect of thymol and thymol acetate on sheep gastrointestinal nematodes and their toxicity in mice. *Revista Brasileira de Parasitologia Veterinária*, *26*, 323–330. https://doi.org/10.1590/S1984-29612017056.
4. Arshad, Z., Asif Hanif, M., Khan Qadri, W., R., and, & Mumtaz Khan, M. (2014). Role of essential oils in plant diseases protection - A review. *IJCBS*, *6*, 11–17.
5. Bajpai, V. K., Baek, K. H., & Kang, S. C. (2012). Control of *Salmonella* in foods by using essential oils: A review. *Food Research International*, *45*, 722–734. https://doi.org/10.1016/j.foodres.2011.04.052.
6. Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils - A review. *Food and Chemical Toxicology*, *46*, 446–475. https://doi.org/10.1016/j.fct.2007.09.106.
7. Bakkali, F., Averbeck, S., Averbeck, D., Zhiri, A., & Idaomar, M. (2005). Cytotoxicity and gene induction by some essential oils in the yeast Saccharomyces cerevisiae. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *585*, 1–13.
8. Balardin, R. S., & Kelly, J. D. (1997). Re-characterization of *Colletotrichum lindemuthianum* races. *Bean Improvement Cooperative Annual Report*, *40*, 126–127.

9. Ballaré, C. L. (2014). Light Regulation of Plant Defense. *Annual Review of Plant Biology*, *65*, 335–363. https://doi.org/10.1146/annurev-arplant-050213-040145.
10. Blazquez, M. A. (2014). Role of natural essential oils in sustainable agriculture and food preservation. *J Sci Res Rep*, *3*, 1843–1860. http://dx.doi.org/10.9734/JSRR/2014/11376.
11. Brilli, F., Loreto, F., & Baccelli, I. (2019). Exploiting plant volatile organic compounds (VOCs) in agriculture to improve sustainable defense strategies and productivity of crops. *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.00264.
12. Briosi, G., & Cavara, F. (1889). I Fungi Parassiti della Piante Coltivate od utili essicati, delineati e descritti. *Fasc. II*, *nos*. 26–50.
13. Butnariu, M., & Sarac, I. (2018). Essential oils from plants. *Journal of Biotechnology and Biomedical Science*, *1*, 35–43. http://dx.doi.org/10.14302/issn.2576-6694.jbbs-18-2489.
14. Costa, B. H. G., de Resende, M. L. V., Monteiro, A. C. A., Júnior, R., Botelho, P. M., D. M. dos S., & Silva, B. M. (2017). da. Potassium phosphites in the protection of common bean plants against anthracnose and biochemical defence responses. *Journal of Phytopathology*, *166*, 95–102. https://doi.org/10.1111/jph.12665.
15. Da Silva, A. C., de Souza, P. E., Pinto, J. E. B. P., da Silva, B. M., Amaral, D. C., & de Arruda Carvalho, E. (2012). Essential oils for preventative treatment and control of Asian soybean rust. *European Journal of Plant Pathology*, *134*, 865–871.
16. Daferera, D. J., Ziogas, B. N., & Polissiou, M. G. (2003). The effectiveness of plant essential oils on the growth of Botrytis cinerea, Fusarium sp. and Clavibacter michiganensis subsp. michiganensis. *Crop Protection*, *22*, 39–44. http://dx.doi.org/10.1016/S0261-2194(02)00095-9.
17. Dwivedy, A. K., Kumar, M., Upadhyay, N., Prakash, B., & Dubey, N. K. (2016). Plant essential oils against food borne fungi and mycotoxins. *Current Opinion in Food Science*, *11*, 16–21. https://doi.org/10.1016/j.cofs.2016.08.010.
18. FAO (2024). Statistical database. Food and Agriculture Organization of the United Nations. Retrieved June 12, 2024, from https://www.fao.org/faostat/en/#home.
19. Faostat (2024, June 12). Food and agriculture organization of the united nations statistics. Retrieved June 12, 2024, from http://www.fao.org/faostat/en/#data/QC.
20. Fisher, M. C., Hawkins, N. J., Sanglard, D., & Gurr, S. J. (2018). Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science*, *360*, 739–742. https://doi.org/10.1126/science.aap7999.
21. Gadaga, S. J. C., Siqueira, C. S., & Machado, J. C. (2020). Transmission potential of *Colletotrichum lindemuthianum* (race 65) in association with bean seeds under controlled conditions. *Journal of Seed Science*, *42*, e202042025. https://doi.org/10.1590/2317-1545v42231421.
22. Gauillard, F., Richardforget, F., & Nicolas, J. (1993). New spectrophotometric assay for polyphenol oxidase activity. *Analytical Biochemistry*, *215*, 59–65. https://doi.org/10.1006/abio.1993.1554.
23. Godinez-Vidal, D., Rocha-Sosa, M., Sepúlveda-García, E. B., Lara-Reyna, J., Rojas-Martínez, R., & Zavaleta-Mejía, E. (2007). Phenylalanine ammonia lyase activity in chilli CM-334 infected by

Phytophthora capsici and *Nacobbus aberrans*. *European Journal of Plant Pathology*, *120*, 299–303. https://doi.org/10.1007/s10658-007-9215-8.

24. Godoy, C. V., Carneiro, S. M. T. P. G., Iamauti, M. T., Dalla Pria, M., Amorim, L., Berger, R. D., & Bergamin Filho, A. (1997). Diagrammatic scales for bean disease: development and validation. *Zeitschrift für Pflanzenkrankheiten und Pfanzenschutz*, *104*, 336–345.
25. Hendges, C., Stangarlin, J. R., Zamban, V. C., Kuhn, O. J., Tartaro, E. L., & Carmelo, D. B. (2023). Antifungal activity against *Alternaria solani* and control of early blight in tomato by essential oil of citronella. *Rev Ceres*, *70*, 101–111. https://doi.org/10.1590/0034-737X202370020012.
26. Hoyos, J. M. Á., Alves, E., Rozwalka, L. C., Souza, E. A., & Zeviani, W. N. (2012). Antifungal activity and ultrastructural alterations in *Pseudocercospora griseola* treated with essential oils. *Ciência e Agrotecnologia*, *36*, 270–284. https://doi.org/10.1590/S1413-70542012000300002.
27. Jamiołkowska, A. (2020). Natural compounds as elicitors of plant resistance against diseases and new biocontrol strategies. *Agronomy*, *10*, 173. https://doi.org/10.3390/agronomy10020173.
28. Junqueira, N. T. V., Chaves, G. M., Zambolim, L., Romeiro, R. S., & Gasparotto, R. (1984). Isolamento, cultivo e esporulação de *Microcyclus ulei*, agente etiológico do mal das folhas da seringueira. *Revista Ceres*, *31*, 322–331.
29. Khan, M. S. A., & Ahmad, I. (2011). *vitro* antifungal, anti-elastase and anti- keratinase activity of essential oils of *Cinnamomum*, *Syzygium* and *Cymbopogon*-species against *Aspergillus fumigatus* and *Trichophyton rubrum*. *Phytomedicine*, *19*, 48–55. https://doi.org/10.1016/j.phymed.2011.07.005.
30. Li, W., Yuan, S., Sun, J., Li, Q., Jiang, W., & Cao, J. (2018). Ethyl *p*-coumarate exerts antifungal activity in vitro and in vivo against fruit *Alternaria alternata* via membrane-targeted mechanism. *International Journal of Food Microbiology*, *278*, 26–35. https://doi.org/10.1016/j.ijfoodmicro.2018.04.024.
31. Lopez-Reyes, J. G., Spadaro, D., Prelle, A., Garibaldi, A., & Gullino, M. L. (2013). Efficacy of plant essential oils on postharvest control of rots caused by fungi on different stone fruits *in vivo*. *Journal of Food Protection*, *76*, 631–639. https://doi.org/10.4315/0362-028x.jfp-12-342.
32. Maguire, J. D. (1962). Speed of germination-aid in selection and evaluation for seedling emergence and vigor. *Crop Science*, *2*, 176–177. https://doi.org/10.2135/cropsci1962.0011183X000200020033x.
33. Mori, T., Sakurai, M., & Sakuta, M. (2001). Effects of conditioned medium on activities of PAL, CHS, DAHP synthase (DS-Co and DS-Mn) and anthocyanin production in suspension cultures of *Fragaria ananassa*. *Plant Science*, *160*, 355–360. https://doi.org/10.1016/S0168-9452(00)00399-X.
34. OMRI (2024). Products list, Crop products. 1-379. Retrieved June 30, 2024, from https://www.omri.org/sites/default/files/opl_pdf/CompleteCompany-NOP-EN.pdf.
35. Padder, B. A., Sharma, P. N., Awale, H. E., & Kelly, J. D. (2017). *Colletotrichum lindemuthianum*, the causal agent of bean anthracnose. *Journal of Plant Pathology*, *99*, 317–330. http://dx.doi.org/10.4454/jpp.v99i2.3867.

36. Pane, C., Villecco, D., Roscigno, G., Falco, E. D., & Zaccardelli, M. (2013). Screening of plant-derived antifungal substances useful for the control of seedborne pathogens. *Archives of Phytopathology And Plant Protection*, *46*, 1533–1539. https://doi.org/10.1080/03235408.2013.771458.

37. Parikh, L., Agindotan, B. O., & Burrows, M. E. (2021). Antifungal activity of plant-derived essential oils on pathogens of pulse crops. *Plant Disease*. https://doi.org/10.1094/PDIS-06-20-1401-RE. PDIS–06-20-1401.

38. Perina, F. J., Amaral, D. C., Fernandes, R. S., Labory, C. R., Teixeira, G. A., & Alves, E. (2014). Thymus vulgaris essential oil and thymol against *Alternaria alternata* (Fr.) Keissler: effects on growth, viability, early infection and cellular mode of action. *Pest Management Science*, *71*, 1371–1378. https://doi.org/10.1002/ps.3933.

39. Pinto, J. M. A., Pereira, R., Mota, S. F., Ishikawa, F. H., & Souza, E. A. (2012). Investigating phenotypic variability in *Colletotrichum lindemuthianum* populations. *Phytopathology*, *102*, 490–497. https://doi.org/10.1094/phyto-06-11-0179.

40. Regnier, T., Combrinck, S., Veldman, W., & Du Plooy, W. (2014). Application of essential oils as multi-target fungicides for the control of *Geotrichum citri-aurantii* and other postharvest pathogens of citrus. *Industrial Crops and Products*, *61*, 151–159. https://doi.org/10.1016/j.indcrop.2014.05.052.

41. Rozwalka, L. C., Alves, E., & Amaral, D. C. (2010). Ultrastructural study of conidia of *Colletotrichum gloeosporioides* and *Colletotrichum musae* treated with essential oils. *Interciencia*, *35*, 912–915.

42. Sadgrove, N., & Jones, G. (2015). A Contemporary introduction to essential oils: chemistry, bioactivity and prospects for Australian agriculture. *Agriculture*, *5*, 48–102. https://doi.org/10.3390/agriculture5010048.

43. Saravanan, T., Bhaskaran, R., & Muthusamy, M. (2004). *Pseudomonas fluorescens* induced enzymological changes in banana roots (Cv. *Rasthali*) against *Fusarium* wilt. *Plant Pathololgy Journal*, *3*, 72–80. http://dx.doi.org/10.3923/ppj.2004.72.80.

44. Sarto, M. P. M., & Zanusso Júnior, G. (2014). Atividade antimicrobiana de óleos essenciais. *Revista UNINGÁ Review*, *20*, 98–102.

45. Shaner, G., & Finney, R. F. (1977). The effect of nitrogen fertilization on the expression of slow-mildewing resistance in knox wheat. *Phytopathology*, *67*, 1051–1056. https://doi.org/10.1094/Phyto-67-1051.

46. Sil, A., Pramanik, K., Samantaray, P., Mondal, M. F., & Yadav, V. (2020). Essential oils: A boon towards eco-friendly management of phytopathogenic fungi. *Journal of Entomology and Zoology Studies*, *8*, 1884–1891.

47. Singh, G., Singh, O. P., Rao, G. P., & Sharma, S. R. (2002). The vapour action of essential oils and monoterpenoids against pathogenic fungi. *Sugar Tech*, *4*, 69–71. https://doi.org/10.1007/BF02956885.

48. Soković, M. D., Vukojević, J., Marin, P. D., Brkić, D. D., Vajs, V., & van Griensven, L. J. L. D. (2009). Chemical composition of essential oils of Thymus and Mentha species and their antifungal activities. *Molecules*, 238–249. https://doi.org/10.3390%2Fmolecules14010238.

49. Soković, M., & van Griensven, L. J. L. D. (2006). Antimicrobial activity of essential oils and their components against the three major pathogens of the cultivated button mushroom, *Agaricus bisporus*. *European Journal of Plant Pathology*, *116*, 211–224. https://doi.org/10.1007/s10658-006-9053-0.
50. Soylu, E. M., Soylu, S., & Kurt, S. (2006). Antimicrobial activities of the essential oils of various plants against tomato late blight disease agent *Phytophthora infestans*. *Mycopathologia*, *161*, 119–128. https://doi.org/10.1007/s11046-005-0206-z.
51. Stenger, L. D., Libardoni, G., Junior, A. W., Zanela, J., et al. (2021). Essential oils in pathogen resistance induction of *Eucalyptus benthamii* Maiden et Cambage. *Ciência Rural*, *51*, e20190915. https://doi.org/10.1590/0103-8478cr20190915.
52. Urbanek, H., Kuznial-Gebarowska, E., & Herk, H. (1991). Elicitation of defence responses in bean leaves by *Botrytis cinerea* polygalacturonase. *Acta Physiololgy Plant*, *13*, 43–50.
53. Walling, L. L. (2001). Induced resistance: from the basic to the applied. *Trends Plant Science*, *6*, 445–447. https://doi.org/10.1016/S1360-1385(01)02046-5.
54. Wang, D., Zhang, J., Jia, X., Xin, L., & Zhai, H. (2019). Antifungal effects and potential mechanism of essential oils on *Colletotrichum gloeosporioides in vitro* and *in vivo*. *Molecules*, *24*, 3386. https://doi.org/10.1016/j.indcrop.2022.115975.
55. Wirth, S. J., & Wolf, G. A. (1990). Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. *Journal of Microbiological Methods*, *12*, 197–205. https://doi.org/10.1016/0167-7012(90)90031-Z.
56. Wu, T., Zhang, B., Luo, X., Li, A., Zhang, S., An, J., Zhang, Z., & Liu, Y. (2023). Antifungal efficacy of sixty essential oils and mechanism of oregano essential oil against *Rhizoctonia solani*. *Industrial Crops and Products*, *191*, 115975. https://doi.org/10.1016/j.indcrop.2022.115975.
57. Yan, J., Wu, H., Chen, K., Feng, J., & Zhang, Y. (2021). Antifungal activities and mode of action of *Cymbopogon citratus*, *Thymus vulgraris*, and *Origanum heracleoticum* essential oil vapors against *Botrytis cinerea* and their potential application to control postharvest strawberry gray mold. *Foods*, *10*, 2451. https://doi.org/10.3390/foods10102451.
58. Yapryntseva, M. A., Zhivotovsky, B., & Gogvadze, V. (2024). Permeabilization of the outer mitochondrial membrane: Mechanisms and consequences. Biochimica et Biophysica Acta (BBA) -. *Molecular Basis of Disease*, *1870*, 167317.
59. Zaker, M. (2016). Natural plant products as eco-friendly fungicides for plant diseases control- a review. *The Agriculturists*, *14*, 134. http://dx.doi.org/10.3329/agric.v14i1.29111.
60. Zheng, C. M., Teng, B., Gao, F. L., & Wu, Z. P. (1999). Studies on the changes of superoxido dismutase, peroxidase and poliphenol oxidase in seed coat of soybeans after infection with soybean mosaic virus. *Scientia Agricultura Sinica*, *32*, 99–101.

Figures

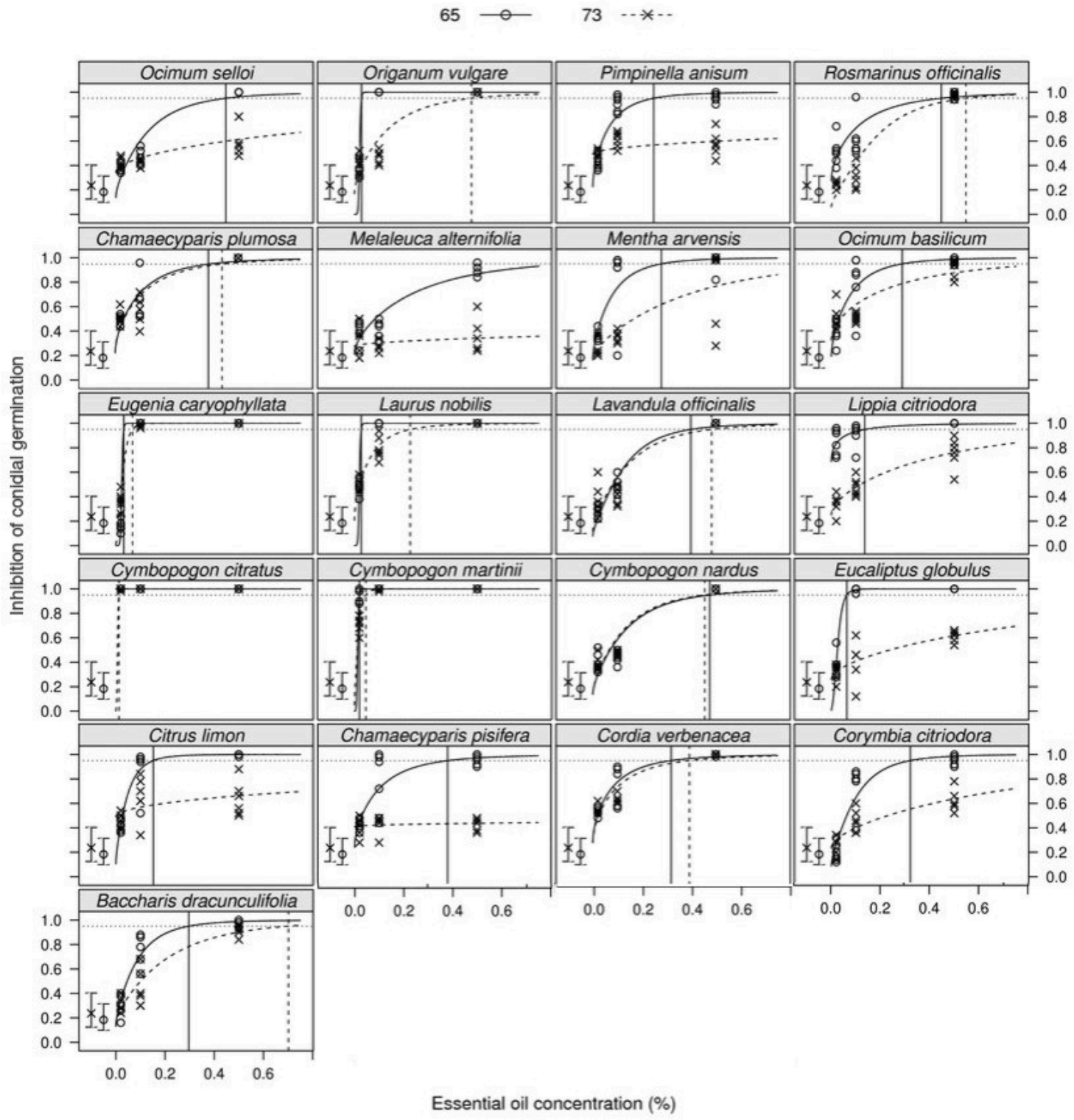

Figure 1

Regression models fitted to data of number of germinated conidia. Vertical dashed lines represent the concentration that causes 95% germination inhibition in races 65 (o) and 73 (x) of *Colletotrichum lindemuthianum*. Confidence intervals at 95% for germination rate in control treatments are shown near the regression curves. Dots stand for germination rate.

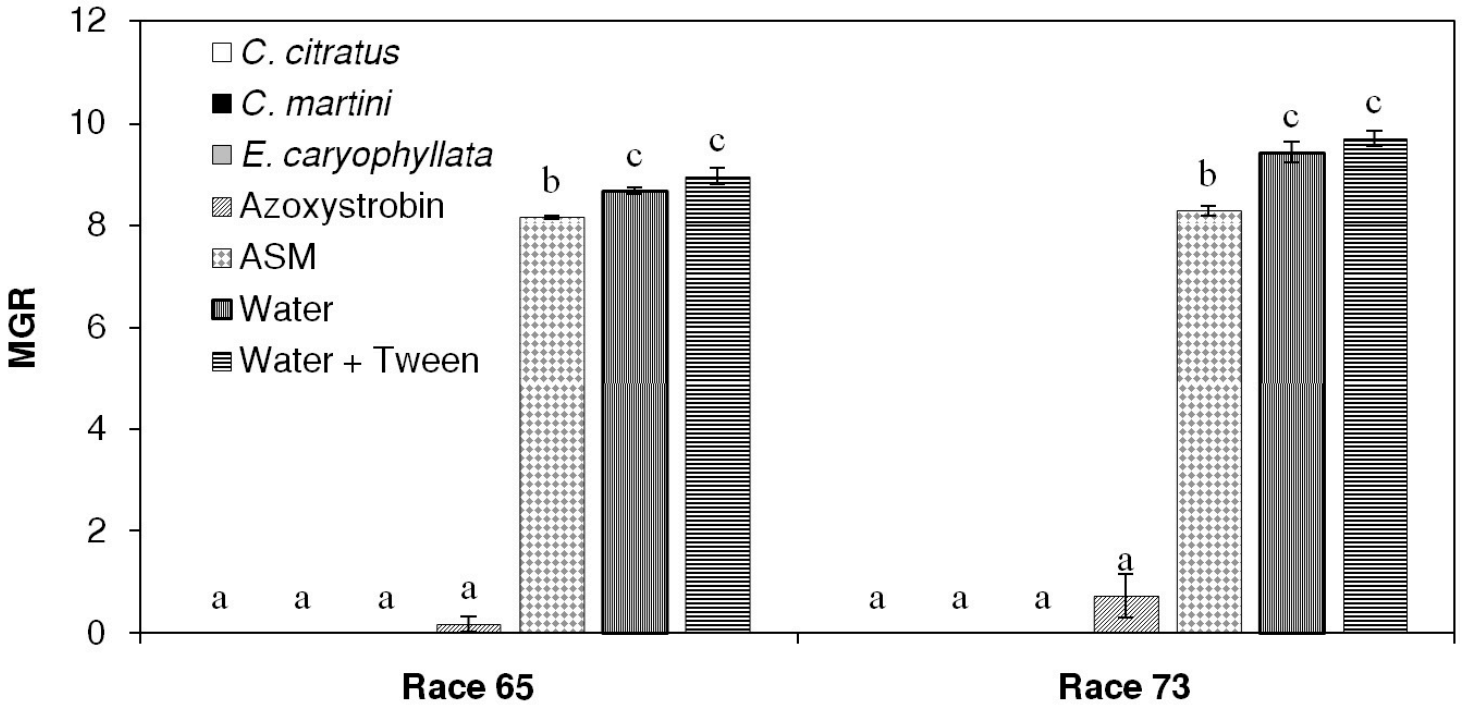

Figure 2

Effect of *C. citratus, C. martini* and *E. caryophyllata* oils at 0.1%, azoxystrobin fungicide 0.4 g L^{-1}, acibenzolar-S-methyl (ASM) 0.08 g L^{-1}, distilled water and distilled water plus Tween 20, 0.1% in mycelial growth rate (MGR) in two races of *Colletotrichum lindemuthianum*. Means followed by the same letter in each race do not differ by *Scott Knott test* ($p \leq 0.05$). Error bars represent standard error.

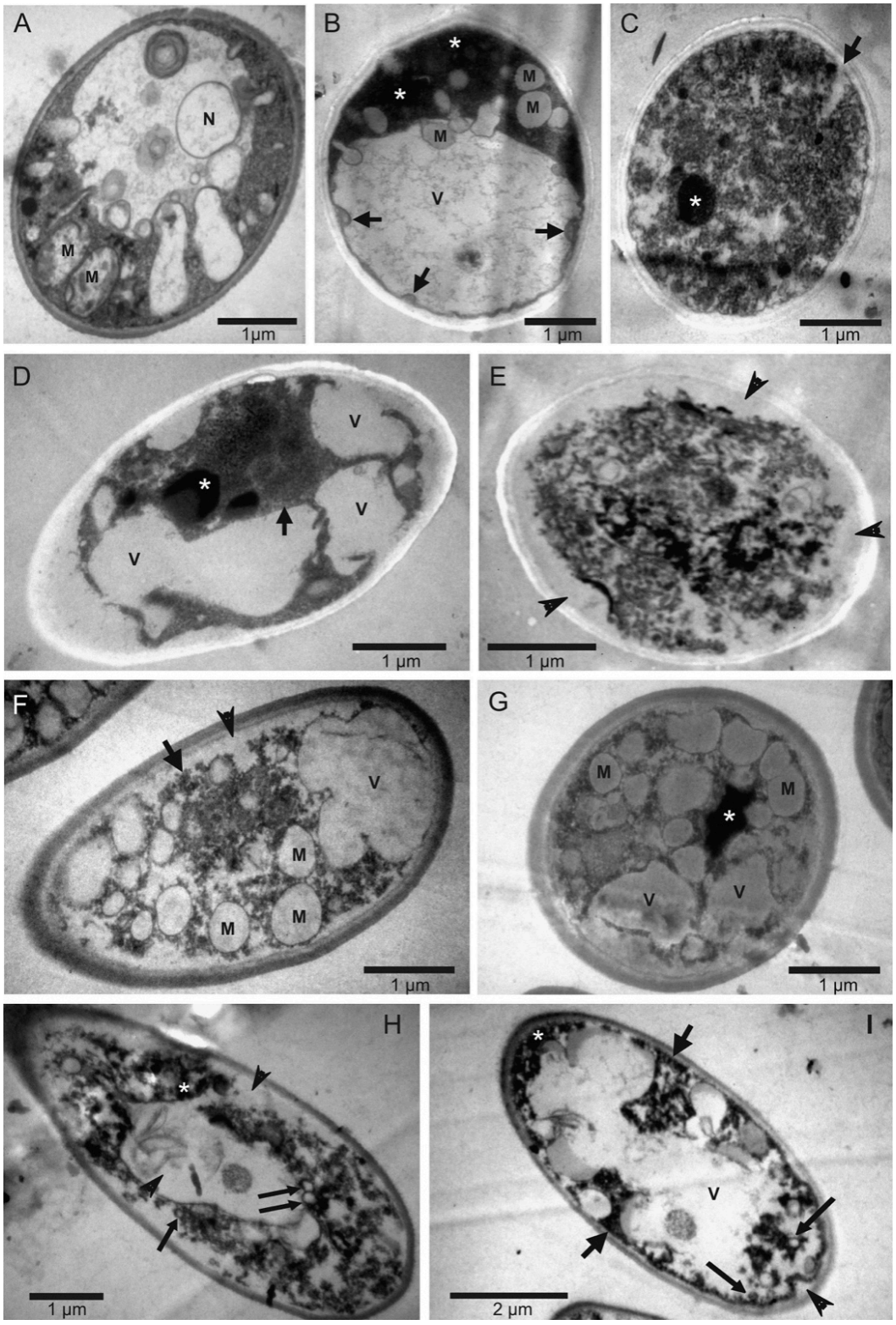

Figure 3

Transmission electron micrographs of the effect of essential oils (EOs) 0.1% on conidial ultrastructure of *Colletotrichum lindemuthianum* race 65. **A**: Control, mitochondria (M), nucleus (N). **B** and **C**: fungicide treatment. **B**: some mitochondria (M), a large vacuole (V), invagination of the plasmalemma (arrows) and electro-dense material (asterisks). **C**: severely damaged conidia with broken plasmalemma (arrow) and electro-dense material (asterisks). **D** and **E**: treatment with essential oil of *Cymbopogon citratus*. **D**:

intense vacuolization (V), cytoplasmic condensation (arrows) and electro-dense material (asterisks). **E**: very degenerate conidia, probably not feasible, with plasmolysis and voids (arrowheads). **F** and **G**: treatment with essential oil of *Eugenia caryophyllata*. **F**: cytoplasmic condensation (arrows), voids (arrowheads), vacuoles (V) and several mitochondria (M). **G**: some mitochondria (M), vacuoles (V) and electro-dense material (asterisks). **H** and **I**: treatment with essential oil of *Cymbopogon martini*. **H**: altered conidia with vesicles (arrows) and voids (arrowheads). **I**: invagination of plasma membrane (arrowheads), vesicles (thin arrows), vacuoles (V), electro-dense material (asterisks) and dispersed cytoplasm mixed with voids or vacuoles (larger arrows).

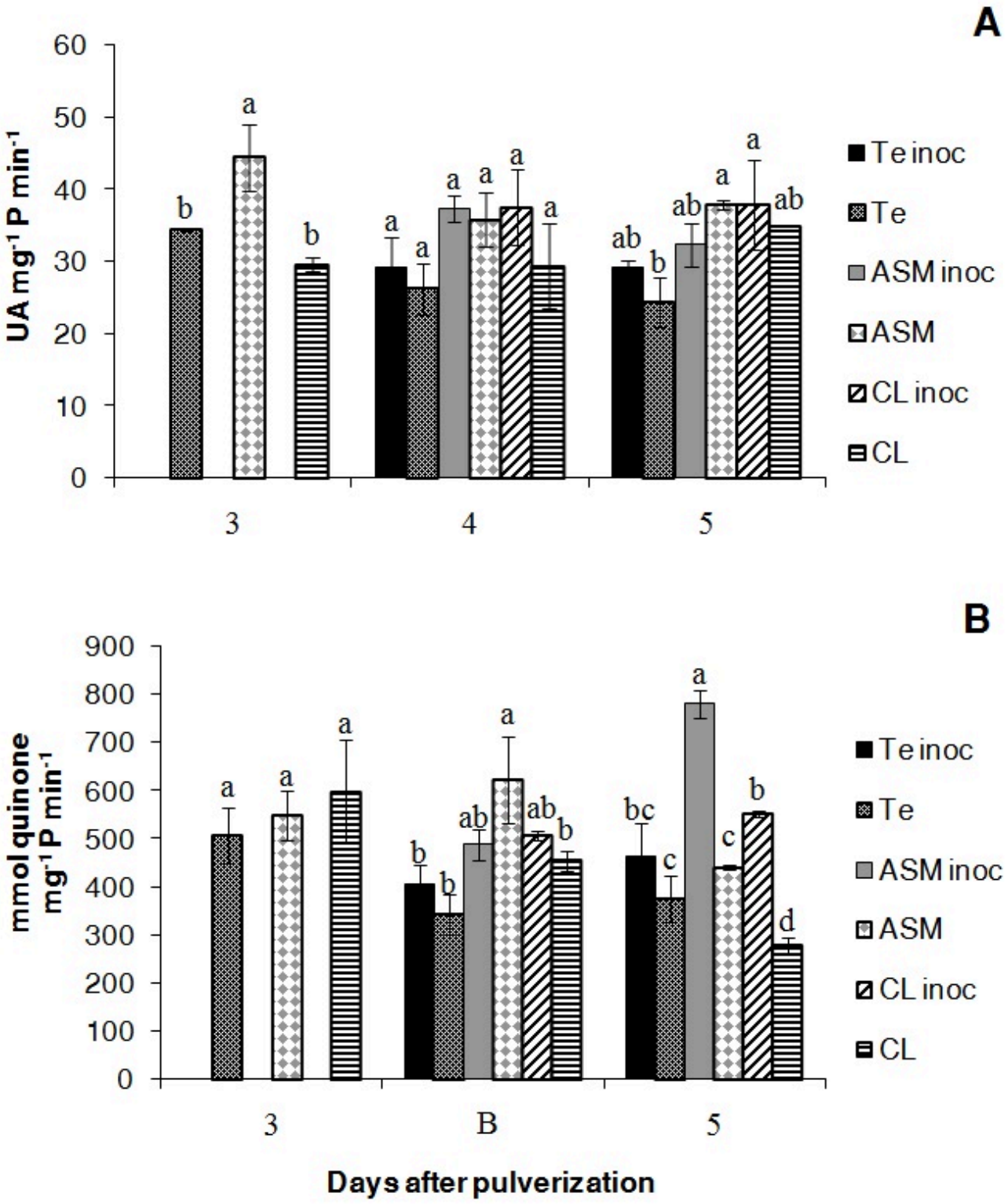

Figure 4

Activity of chitinase (CHI) (A) and polyphenol oxidases (PPO) (B) in leaves of bean cv. Pérola after spraying with water (Te), water inoculated with *C. lindemuthianum* (Te inoc), acibenzolar S-methyl (ASM), acibenzolar S-methyl inoculated with *C. lindemuthianum* (ASM inoc), *C. citratus* (Cc) and *C. citratus* inoculated with *C. lindemuthianum* (Ccinoc). Inoculation with *C. lindemuthianum*occurred at 3 days after

spraying. Bars with the same letter do not differ by *Duncan test* ($p \leq 0.05$). Error bars indicate the standard error.

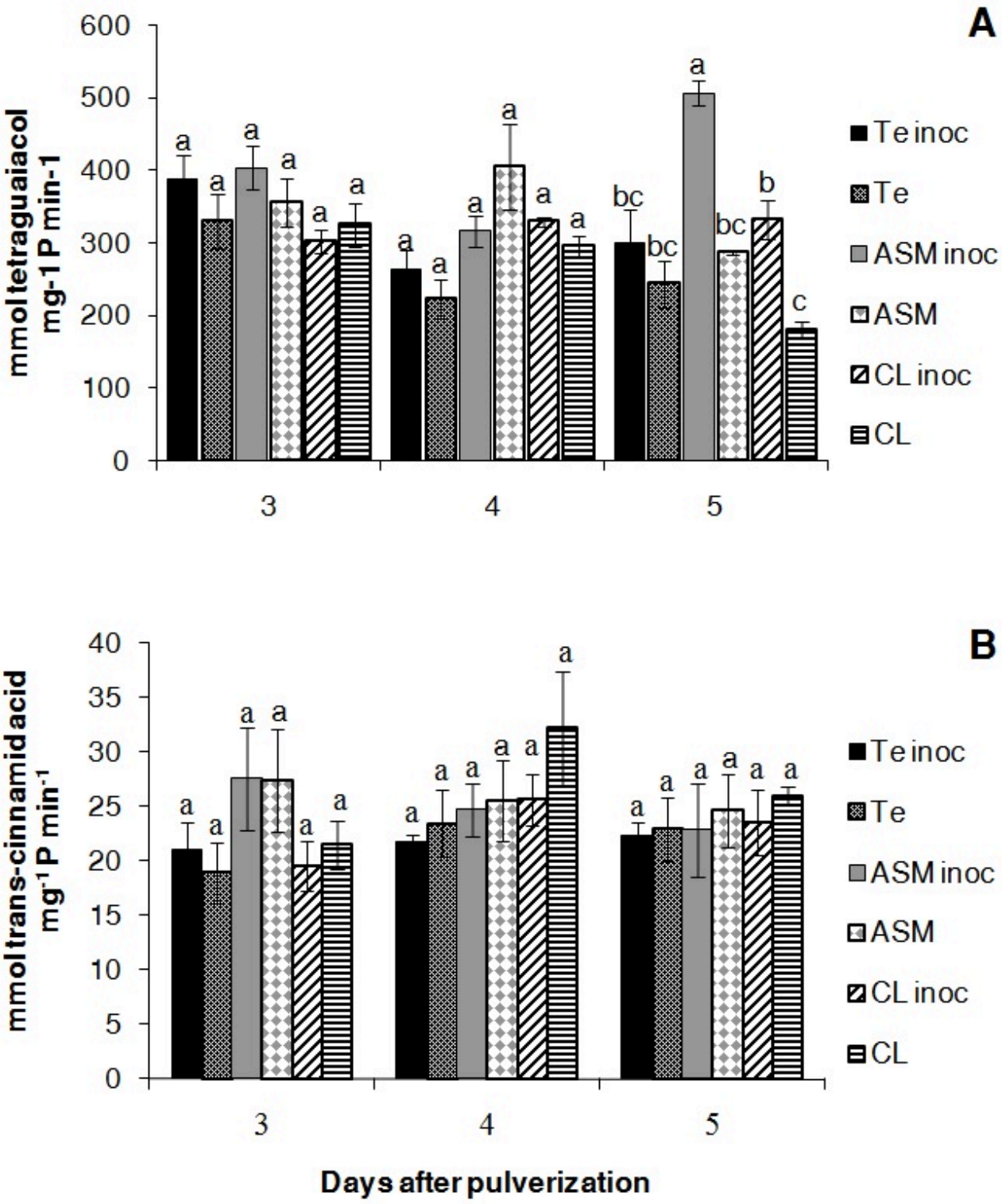

Figure 5

Activity of peroxidase (POX) (A) and phenylalanine ammonia lyase (PAL) (B) in leaves of bean cv. Pérola after spraying with water (Te), water inoculated with *Colletotrichum lindemuthianum* (Te inoc), acibenzolar S-methyl (ASM), acibenzolar S-methyl inoculated with *C. lindemuthianum* (ASM inoc), *C.*

citratus (Cc) and *C. citratus*inoculated with *C.lindemuthianum*(Ccinoc). Inoculation with *C. lindemuthianum* occurred three days after spraying. Bars with the same letter do not differ by *Duncan test* ($p \leq 0.05$). Error bars indicate the standard error.