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Essential oil of *Cymbopogon flexuosus*, *Vernonia polyanthes* and potassium phosphite in control of bean anthracnose

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This study evaluated essential oils of *Cymbopogon flexuosus* (EOC), *Vernonia polyanthes* (EOV), and potassium phosphite (PP) in control of bean anthracnose caused by *Colletotrichum lindemuthianum*. This study assessed mycelial growth and conidial germination *in vitro*, scanning electron microscopy (SEM), and enzymes peroxidase (POX) and phenylalanine ammonia-lyase (PAL). EOC and EOV in doses 1,450 and 1,320 µL L^{-1} reduced disease severity by 57.2 and 37.6%, respectively. Major components identified in EOC were geraniol 46.8% and nerol 33.4%, and germacrene-D (42.2%) and bicyclogermacrene (17.2%) in EOV. SEM images showed that PP, EOC and EOV reduced mycelial growth and emission of germ tubes. PP and EOC increased POX and PAL rates in bean tissue. Bean anthracnose was controlled by direct antifungal activity of PP and EOC and induction of defense enzymes

Key words: Alternative disease control, *Colletotrichum lindemuthianum*, resistance induction, scanning electron microscopy, *Phaseolus vulgaris*.

INTRODUCTION

Anthracnose caused by *Colletotrichum lindemuthianum* (Sacc & Magnus) is a major destructive disease of common bean (*Phaseolus vulgaris* L.). The seed borne fungi has high pathogenic variability with over 100 described strains (Campa et al., 2011). In Brazil, infected seeds may cause up to 100% losses in bean yield under favorable climatic conditions (Damasceno et al., 2007).

The most economically viable and effective strategy for disease control is by using bean cultivars resistant to fungi (Ishikawa et al., 2008). However, this kind of management is difficult to implement due to the high pathogenic variability of *C. lindemuthianum* (Ishikawa et al., 2012). Consequently, synthetic fungicides are widely used by farmers to control bean anthracnose

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(Gillard et al., 2012). Short term use of these products effectively helps the farmer achieve high yields. Thus, several research groups have sought cheaper and low-toxicity alternatives for controlling fungi pathogenic to plants. Essential oils (EOs) and potassium phosphite (PP) are potentially useful both for their direct toxicity for fungi, which inhibit mycelial growth and spore germination, as for induction of phytoalexins, which indicate the presence of elicitor compounds (Pereira et al., 2012).

This study assessed essential oils of *Cymbopogon flexuosus* (EOC), *Vernonia polyanthes* (EOV), and potassium phosphite (PP) to control of bean anthracnose caused by *C. lindemuthianum*.

METHODOLOGY

Essential oil extraction

Leaves of *C. flexuosus* and *V. polyanthes* were collected in the season of winter on the month August in the city of Lavras. Essential oils were extracted from 1000 g fresh leaves through steam distillation for 90 min, using a Marconi MA480 stainless steel distiller. The aqueous phase was extracted with dichloromethane (3 × 25 ml). The organic phase was dried with anhydrous magnesium sulfate, filtered and the solvent evaporated until dryness.

Essential oil analysis

Analysis of chemical composition was performed according to Rosado et al. (2013). Oil quantitative analysis was performed using gas-phase chromatography coupled to a hydrogen flame ionization detector (GC-FID) on Agilent® 7890. A system equipped with HP-5 fused in the electron impact ionization mode (70 eV), injector split, capillary colum HP-5 (30 m × 0.25 mm × 0.25 µm). Temperature: injector = 220°C, collum = 60°C for 1.5 min, ramp = 3°C for min. Carrier gas He = 1 ml min^{-1}.

Retention indices (RI) have been obtained according to the method of Van den Dool and Dec Kratz (1963).

Analysis of mycelial growth inhibition in *C. lindemuthianum*

Pure culture of *C. lindemuthianum,* strain 65, isolate LV 175 was provided by the Laboratory of Plant Resistance, Department of Biology, UFLA. The single spore culture was obtained. In inhibition test of mycelial growth, the treatments were EOC and EOV at concentrations of 125, 250, 500, 1000, and 2000 ml L^{-1}, PP at a dose of 5 ml L^{-1} and trifloxystrobin + fungicide at a dose of 0.75 ml L^{-1}. All treatments were added to potato dextrose Agar (PDA) medium containing Tween 20 (0.1%). A treatment containing Tween 20 (0.1%) was used to isolate its effect and treatment with sterile distilled water as negative control. After solidification, mycelial disks (8 mm) of *C. lindemuthianum* were placed in the center of petri dishes (90 mm) with the treatments described earlier and incubated at 25°C under photoperiod of 12 h.

Evaluations were performed daily until fungal colonies covered 2 thirds of the medium surface. Percentage of growth inhibition (PGI) was calculated using the formula: PGI = [(diameter of control - diameter of treatment) / diameter of control] × 100 for each treatment as compared to the control. The experiment was conducted in completely randomized design with six replicates, each plot consisting of a petri dish.

Evaluation of conidial germination

In this study, suspensions of *C. lindemuthianum* conidia growing at PDA medium in test tubes were used. Sterile distilled water (10 ml) was added into the test tubes and they were agitated. The suspension obtained was filtered with sterile gauze and its concentration was adjusted to 1.15×10^5 conidia ml^{-1} in a Neubauer chamber. One milliliter spore suspension and 1 ml of the treatments described earlier were spread over the surface of petri dishes (60 mm) with the water ágar medium at 2% with handles Drigalski. Then the plates were incubated at 25°C under a 12-h photoperiod. After 24 h, lactoglycerol was used for stopping the germination process.

Percentage of growth inhibition (PGI) was evaluated by counting the number of germinated and non-germinated conidia. PGI was obtained using the formula: PGI = [(number of germinated conidia control - number of conidia in the treatment) / number of conidia in the control] × 100 for each treatment as compared to the control. The experiment was conducted in completely randomized design with six replicates, each plot consisting of a petri dish.

Evaluation of treatments in anthracnose control

For evaluation of treatments in anthracnose control bean seedlings of cultivar Pearl was used. Twenty one days after sowing, the treatments were performed. 48 h after application, the plants were sprayed with a suspension of inoculum of 1×10^6 and incubated in a humid chamber for 14 h. The experiment was conducted in a randomized block design with four replications. Each plot consisted of two pots containing three plants each.

Anthracnose severity was assessed every seven days through a rating scale proposed by Godoy et al. (2006). The area under the disease progress curve (AUDPC) was calculated for each treatment, according to Shaner and Finney (1977).

Enzymatic activity of peroxidase (POX) and phenylalanine ammonia lyase (PAL)

To determine the enzymatic activity of peroxidase (POX) and phenylalanine ammonia-lyase (PAL), the most promising treatments were selected. Distilled water was used as negative control. Bean plants at the stage V3/V4 were sprayed after 7 days and were inoculated as previously described. The experiment was a randomized complete block design with three replications and the portion composed of three plants.

Bean leaf samples were collected on days 1, 3, 6, 8, 10 and 13 after spraying treatments. The samples were stored at -80°C for later analysis.

To determine enzymatic 0.2 g of leaf tissue with 1% polyvinylpyrrolidone and liquid N_2 were ground in a mortar. It was homogenized in 1.3 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM phenylmethylsulfonyl fluoride, and was centrifuged at 11,000 ×g for 30 min at 4°C and the supernatant was used for enzymatic determination.

The POX activity was determined by oxidation of guaiacol according to the method of Kar and Mishra (1976). Ninety microliters of potassium phosphate buffer 100 mM (pH 7.0), 30 µl of guaiacol 40 mM and 25 µl of H_2O_2 125 mM were added to 10½ extract. Absorbance was measured at 420 nm in ELISA reader every 10 s for 2 min after adding the sample to the mixture. Molar extinction coefficient of 1.24 mM cm^{-1} was used to calculate the POX activity (Maehly and Chance, 1955), which was expressed in mM purpurogallin produced min^{-1} mg^{-1} protein.

The PAL activity was measured by adding 40 µ to a mixture containing 110 µl of 100 mM Tris-HCl (pH 8.8) and 50 µl of 100 mM L-phenylalanine. The reaction mixture was incubated in the ELISA

reader at 37°C for 10 min. The absorbance of derivatives of trans-cinnamic acid was measured in a spectrophotometer at 280 nm and the molar extinction coefficient of 5000 was used mM^{-1} cm^{-1} (Zucker, 1965) for calculating the PAL activity, which was expressed in mM min^{-1}-mg protein. The protein concentration in each sample was determined according to the colorimetric method described by Bradford (1976).

Analysis in scanning electron microscope

Pearl bean cultivar at V3/V4 stage was sprayed with the most promising treatments: OEV and OEC 1000 µl L^{-1} and PP 5 µl L^{-1}. As a negative control, sterile distilled water was sprayed. Two days after application, seven of the third trifoliate leaves were collected and packaged in plastic trays with the bottom of the tray covered with foam rubber and two sheets germitest wetted with distilled water.

Points were marked and 25 µl of suspension with 1.5×10^3 spores of *C. lindemuthianum* ml^{-1} was deposited. After inoculation, the trays were covered with clear plastic and placed in a growth chamber at 25°C until the end of collection.

Samples were collected after 4, 8, 16, and 48 h of inoculation, using 5 mm circular cuts with a scalpel inoculated into each point. The samples were placed Karnovsky's fixative at pH 7.2 and stored at 4°C for 24 h. After this period, the samples were placed in 0.05 M cacodylate tampon and washed three times for 10 min and transferred to osmium tetroxide and 1.0% water for 1 h, washed three times with distilled water.

Then the samples were dehydrated in acetone series (25, 50, 70, 90, and 100% three times) and brought to the critical point dryer Balzers CPD 030 for replacing acetone by CO_2. The samples were mounted on stubs and coated with gold using the Balzers SCD 050 evaporator for observation in a scanning electron microscope LEO EVO 40. The images were digitally generated 20 kV to a 10 mm working distance, then the images were worked in Corel Draw Photo Paint 12.

Statistical analysis

Data were subjected to analysis of variance, and mean values were compared by the Scott Knott test at 5% probability for qualitative factors, while regression analysis was applied for quantitative factors.

RESULTS

Yields and chemical composition

Chemical composition of essential oils of *C. flexuosus* and *V. polyanthes* are as shown in Table 1. Oil yields were 1.27 and 0.02% (v/w), respectively for *C. flexuosus* and *V. polyanthes*.

Twenty compounds were identified in the essential oil extracted from *C. flexuosus* leaves, representing 97.7% of total constituents. Twenty two compounds were identified in *V. polyanthes* leaf essential oil, representing 97.6% of the total constituents.

The composition of *C. flexuosus* essential oil is much simpler and mainly composed by monoterpenes (96.4%) in contrast to *V. polyanthes* essential oil, which is primarily composed of sesquiterpenes (94.3%).

Evaluation of mycelial growth inhibition and conidial germination in *C. lindemuthianum*

Essential oils of *C. flexuosus* and *V. polyanthes* inhibited mycelial growth and conidial germination in *C. lindemuthianum*. Oil of *C. flexuosus* was more efficient, and reduced colony diameter in *C. lindemuthianum* starting from the lowest concentration (125 µl L^{-1}). Total suppression of fungus mycelial growth occurred in concentrations higher than 615 $µl^{-1}$ of EOC (Figure 1). The concentration capable of inhibiting mycelial growth was 50% in 162 µl L^{-1} and 1918 µl L^{-1} in EOC and EOV, respectively.

EOC was more efficient for percentage of conidia germination inhibition, and concentration of 689 µl L^{-1} totally inhibited *C. lindemuthianum* germination (Figure 1C). The concentration capable of inhibiting at least 50% conidial germination was estimated in 220 and 850 µl L^{-1} in EOC and EOV, respectively.

Table 2 compares the effect of essential oil (EO) doses, showing higher antifungal activity with potassium phosphite (PP), fungicide (F), and EOC at 1000 µl L^{-1}. The highest antifungal activity occurred with F and EOC, which completely inhibited mycelial growth and fungal germination.

Essential oils and potassium phosphite in control of bean anthracnose in greenhouse

In greenhouse, essential oils of *C. flexuosus* and *V. polyanthes* reduced the area under the curve of progress of anthracnose (AUCPA). EOC provided greater reduction (57.2%) as compared to the control in concentration 1450 µl L^{-1} whereas EOV provided 37.6% as compared to the control in concentration 1320 µl L^{-1} (Figure 2).

Treatments with potassium phosphite and fungicide reduced AUCPA by 60.4 and 62.1%, respectively, compared to control. There was no statistical difference between PP, F and EOC at 1000 µ L^{-1}.

Studies of germination and mycelial growth *in vivo* in *C. lindemuthianum*

Images of scanning electron microscopy showed no conidia germination in *C. lindemuthianum* in all treatments 4 h after inoculation. Samples collected 8 h after inoculation had no germination either, except for the treatment with distilled water. In this treatment, we observed conidia starting the germination process (Figure 3).

Bean leaves treated with distilled water 16 h after inoculation showed conidia in advanced stage of germination and mycelial growth on the surface of leaves. However, leaves treated with EOC and EOV showed low

Table 1. Relative concentration of constituents of essential oils from fresh leaves of *Cymbopogon flexuosus* and *V. polyanthes.*

Compound	RI[a]	*C. flexuosus*	*V. polyanthes*
α-Pinene (M)	830	-	0.24
β-Pinene (M)	874	-	2.06
Methyl-heptenone (OM)	883	1.01	-
Myrcene (M)	988	0.72	-
β-Terpinene (M)	985	-	0.11
Ocimene (M)	994	-	0.35
β-Ocimene (M)	1020	0.21	-
Linalol (OM)	1098	2.47	-
exo-Isocitral (OM)	1144	0.4	-
Citronelal (OM)	1152	0.21	-
Z-Isocitral (OM)	1164	1.42	-
Not identified m/z=152	1182	2.01	-
Estragole (OM)	1198	0.58	-
N-Decanal (OM)	1205	0.36	-
Neral (OM)	1243	33.40	-
Geraniol (OM)	1254	1.14	-
Geranial (OM)	1273	46.86	-
E-Dimethoxycitral (OM)	1338	-	0.58
Not identified m/z=168	1339	1.01	-
Not identified m/z=168	1375	1.45	-
Cyclosativene (S)	1377	-	2.34
Geranil acetate (OM)	1384	3.08	-
Cycloisosativene (S)	1385	-	1.74
Copaene (S)	1393	-	1.96
trans- Caryophyllene (S)	1420	0.39	-
β-Caryophyllene (S)	1421	-	13.57
Gurjunene (S)	1430	-	0.49
α-Humulene (S)	1455	-	7.84
cis-Cadina-1(6), 4-diene (S)	1462	-	1.96
Ar-Curcumene (S)	1479	0.31	-
Germacrene-D (S)	1484	-	42.18
δ-Curcumene (S)	1496	0.34	-
Bicyclogermacrene (S)	1498	-	17.23
Germacrene A (S)	1506	-	0.77
(E,E)-α-Farnesene (S)	1509	-	0.91
γ-Cadinene (S)	1515	-	0.42
δ-Cadinene (S)	1524	-	1.13
Spathulenol (OS)	1578	-	0.38
Caryophyllene oxide (OS)	1583	0.24	0.84
α-Muurolol (OS)	1642	-	0.22
α-Cadinol (OS)	1655	-	0.20
Total		97.69	97.62

[RI]Retention indices (13). [%]Compounds percentage. [a]Retention indices relative to *n*-alkanes (C_8-C_{20}) on the HP-5 MS Capillary column. M: Monoterpene; OM: oxygenated monoterpene; S: sesquiterpene; OS: oxygenated sesquiterpene.

conidia germination while the treatment with PP had no germination (Figure 3). Large differences were found 48 h after inoculation. Leaves treated with distilled water had clear mycelial growth and emission of numerous germ tubes near ribs in the abaxial surface. Conversely, leaves sprayed with essential oils showed low conidia germination

Table 2. Percentage of inhibition of mycelial growth (PIMG) and conidia germination (PICG) in *Colletotrichum lindemuthianum* subjected to different treatments with essential oils of *C. flexuosus* (EOC) and *V. polyanthes* (EOV), potassium phosphite (PP), fungicide (Trifloxistrobina + Tebuconazol) and control (H_20).

Treatment	PIMG	PICG
PP	100^a	100^a
EOC 1000 µl L^{-1}	100^a	99.13^a
Fungicide	76.08^b	93.3^b
EOV 2000 µl L^{-1}	50.21^c	50.1^c
Tween 20	0.64^d	0.4^d
Control (H_20)	0^d	0^d
CV (%)	5.04	4.52

Means followed by the same letter in the column do not differ statistically by Tukey test at 5% probability. Data were transformed to $\sqrt{x} + 1$.

Table 3. Activity of peroxidase mM min^{-1} mg^{-1} protein in bean plants with and without inoculation of *C. lindemuthianum* at stage V3/V4 collected at 8, 10 and 13 days after spraying with potassium phosphite (PP) 5 ml L^{-1}, essential oil of *C. flexuosus* (EOC) 1000 µl L^{-1} and distilled water (H_2O).

Treatment	Without inoculation			With inoculation		
	8^{NS}	10	13	8	10	13
PP	189.24^a	147.56^a	163.76^a	202.47^{aC}	326.8^{aB}	382.03^{aA}
EOC	118.14^b	134.43^a	150.87^a	192.92^{aB}	334.36^{aA}	315.21^{bA}
H_2O	150.29^b	140.93^a	131.1^a	145.06^{bC}	203.1^{aB}	311.43^{bA}
CV (%)	14.34	-	-	-	-	-

Means followed by the same letter, lowercase in columns and capital letters in lines do not differ by the Scott-Knott test at 5% probability. ^{NS}There was no statistical difference for the factor time of collection x treatments for analysis of non-inoculated plants (p = 0.1815).

and poorly developed mycelium. The treatment with PP had no conidia germination (Figure 3).

Biochemical analysis of induced resistance

For the enzymatic activities, only treatments with EOC and PP in concentration of 1000 µl L^{-1} and 5 ml L^{-1}, respectively were selected.

In inoculated plants, peroxidase (POX) activity was significantly higher in treatment with PP at 8 and 13 days after spraying differing from the control treatment. However, at day 10, there was no significant difference between treatments. Essential oil of *C. flexuosus* provided higher peroxidase activity at day 8 compared to the control. Increase of peroxidase at day 8 in treatments with PP and EOC helped control the pathogen, as inoculation was performed at 7 days after spraying. Plants recognized the fungus and increased the level of POX.

In non-inoculated plants at 8 days after spraying, only PP differed from the other treatments (Table 3). With respect to collection period, there was statistical difference in inoculated plants since PP showed higher content of POX at day 13 compared to days 8 and10. Plants sprayed with EOC had greater activity at 10 and 13 days after spraying. There was a significant difference between inoculated and non-inoculated plants, and plants challenged with fungus *C. lindemuthianum* showed higher levels of POX.

POX activity at 1, 3, 6, 8, 10 and 13 days after spraying in non-inoculated plants (Table 4) suggests that spraying PP had an active role in potentiating and anticipating enzyme activity. This result demonstrates the role of PP in plant defense, since there was difference from the control treatment at 1, 3, 6 and 8 days after spraying. With respect to EOC, there was significant difference compared to control at 1, 3, 6 and 8 days after spraying.

Regarding the activity of the enzyme phenylalanine ammonia lyase (PAL), there was significant difference. In

Table 4. Activity of peroxidase mM min^{-1} mg^{-1} protein in bean plants without *C. lindemuthianum* at stage V3/V4, collected at 1, 3, 6, 8, 10 e 13 days after spraying with potassium phosphite (PP) 5 ml L^{-1}, essential oil of *C. flexuosus* (OEC) 1000 µl L^{-1} and distilled water (H_2O).

Treatment	Days after spraying					
	1	3	6	8	10	13
PP	145.31^{aB}	203.6^{aA}	213.17^{aA}	189.24^{aA}	147.66^{aB}	163.76^{aB}
EOC	151.41^{aB}	193.87^{aA}	185.06^{bA}	118.14^{cB}	134.43^{aB}	150.87^{aB}
H_2O	107.04^{bB}	159.36^{bA}	158.7^{Ba}	150.29^{bA}	141.03^{aA}	131.1^{aB}
CV (%)	11.77	-	-	-	-	-

Means followed by the same letter, lowercase in columns and capital letters in lines do not differ by the Scott-Knott test at 5% probability.

Table 5. Activity of phenylalanine ammonia-lyase mMol min^{-1} mg $protein^{-1}$ in bean plants with and without inoculation of *C. lindemuthianum*, at V3/V4 stage, collected at 8, 10 and 13 days after spraying of potassium phosphite (PP) 5 ml L^{-1}, essential oil of *C. flexuosus* (EOC) 1000 µl L^{-1} and distilled water (H_2O).

Treatment	Without inoculation			With inoculation		
	8	10	13	8	10	13
PP	0.33^{Ab}	1.74^{aA}	0.22^{aB}	1.83^{aB}	2.45^{aA}	0.58^{aC}
EOC	0.25^{aA}	0.59^{bA}	0.55^{aA}	0.94^{bB}	1.98^{bA}	0.80^{aB}
H_2O	0.31^{aB}	0.79^{bA}	0.32^{aB}	0.45^{cA}	0.68^{cA}	0.17^{bC}
CV (%)	24.46	-	-	-	-	-

Means followed by the same letter, lowercase in columns and capital letters in lines do not differ by the Scott-Knott test at 5% probability.

Table 6. Activity of phenylalanine ammonia-lyase (PAL) in mMol min^{-1} mg $protein^{-1}$ in bean plants without inoculation of *C. lindemuthianum*, at V3/V4 stage, collected at 1, 3, 6, 8, 10 and 13 days after spraying of potassium phosphite (PP) 5 ml L^{-1}, essential oil of *C. flexuosus* (EOC) 1000 µl L^{-1} and distilled water (H_2O).

Treatment	Days after spraying					
	1	3	6	8	10	13
PP	1.31^{aC}	2.15^{bA}	1.69^{aB}	0.33^{aD}	1.74^{aB}	0.22^{bD}
EOC	0.73^{bC}	2.53^{aA}	1.44^{bB}	0.25^{aD}	0.59^{bC}	0.55^{aC}
H_2O	0.71^{bA}	0.98^{cA}	0.78^{cA}	0.31^{aB}	0.79^{bA}	0.32^{bB}
CV (%)	13.6	-	-	-	-	-

Means followed by the same letter, lowercase in columns and capital letters in lines do not differ by the Scott-Knott test at 5% probability.

plants inoculated and sprayed with PP, there was greater activity of PAL compared to plants sprayed with EOC and water to 8 and 10 d.a.p. (Table 5). Already at 13 d.a.p., plants sprayed with PP produced less PAL and not different from oil.

Considering PAL activity in non-inoculated plants at 1, 3, 6, 8, 10 and 13 days after spraying, PP was superior to other treatments at days 1, 6 and 10 (Table 6). Plants sprayed with EOC had greater PAL activity at days 3 and 13. There was no significant difference between treatments at day 8. Regarding time of collection, plants sprayed with PP and EOC showed higher PAL activity at day 3. The control treatment had increased PAL production at days 1, 3, 6 and 10.

DISCUSSION

The constituents of *C. flexuoso* in greater quantity were geranial (46.9%) and neral (33.4%), called citral, and

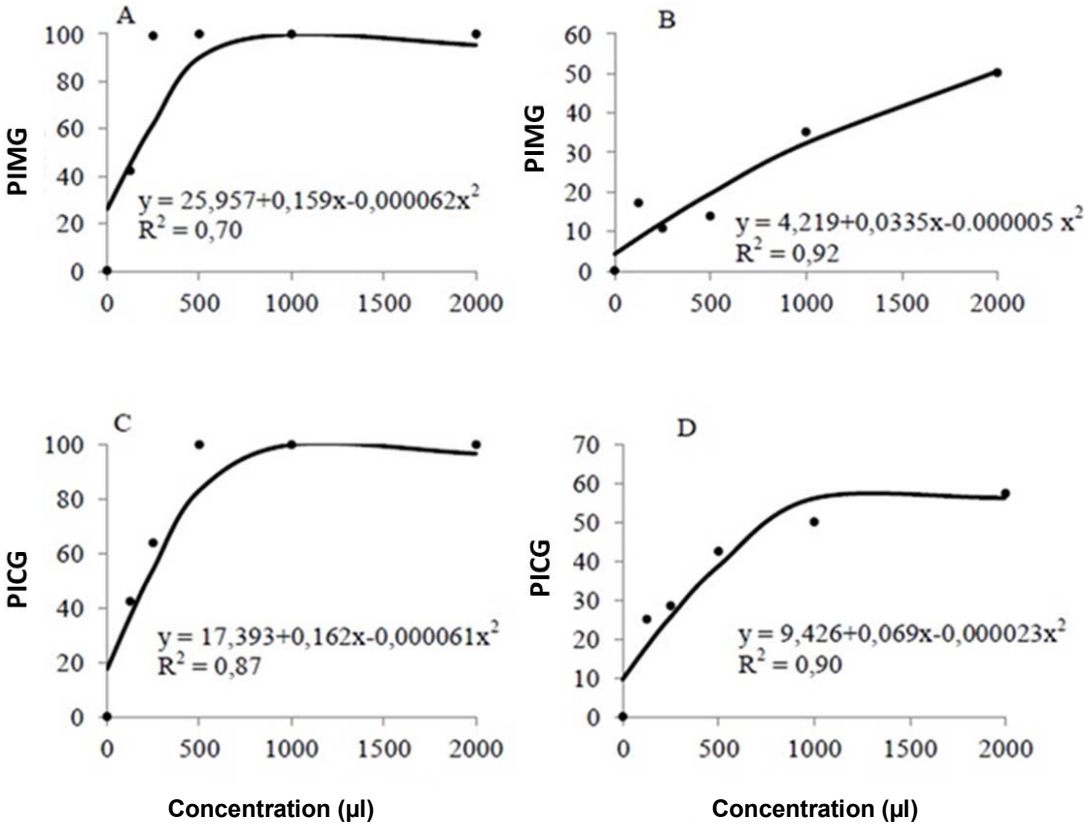

Figure 1. Percentage of inhibition of mycelial growth (PIMG) and conidial germination (PICG) in *C. lindemuthianum* at different concentrations (0, 125, 250, 500, 1000, and 2000 µL L^{-1}) of essential oils of *C. flexuosus* (A and C) and *V. polyanthes* (B and D). Data were transformed to √x + 1.

comprised 80.3% of the essential oil. These results are in agreement with the literature (Adukwu et al., 2012).

The constituents of *V. polyanthes* is mainly composed by germacrene D (42.2%), bicyclogermacrene (17.2%), β-caryophyllene (13.6%) and α-humulene (7.8%), representing 80.8% of total oil. These results are in agreement with the literature (Maia et al., 2010).

Fungicide and fungistatic effect were found when mycelium discs with 100% PIMG were taken from the culture medium at the end of growth analysis and subcultured to PDA plates. Only PP had fungistatic activity, whereas in the other treatments, the effect was fungicidal. This probably resulted from the high volatility of *C. flexuosus* essential oil, which killed fungal cells in the upper part of the mycelium disc that comes not in contact with culture medium.

Anaruma et al. (2010) determined the activity of 28 essential oils from medicinal plants against *Colletotrichum gloeosporioides*. Four species (C. *flexuosus, Cymbopogon citratus, Coriandrum sativum* and *Lippia alba*) showed better antifungal activity. Citral is the main component of essential oils of most species of *Cymbopogon* species, and many authors attribute to this compound the control of plant pathogenic fungi. Valencia et al. (2011) found antifungal effect of essential oil of *C. citratus* on *C. gloeosporioides*. Alzate (2009) demonstrated that citral completely inhibited mycelial growth and sporulation of *C. acutatum* outperforming Mancozeb fungicide. According to Bakkali et al. (2008), as these oxygenated monoterpenes are hydrophobic they will probably want to move towards the aqueous phase of membrane structures. Accumulation of essential oil constituents in the lipid bilayer of the cytoplasmic membrane makes it permeable, thus promoting dissipation of the proton motive force. It also reduces ATP pool, internal pH and electric potential, causing loss of ions such as potassium and phosphate. Thus, damage leads to impairment of membrane functions.

According to Rasooli et al. (2006), when in direct contact with microorganisms, these substances cause permeability of cell membranes and leakage of their contents. Furthermore, terpene alcohols were identified in EOC composition, such as geraniol and linalool (Table 1).

Few studies have reported antimicrobial activity of species of *Vernonia* spp. Essential oil of *V. polyanthes* presented lower antifungal activity than *C. flexuosus* oil (Figure 1B and D). These results demonstrate that microorganisms differ in their resistance to certain essential

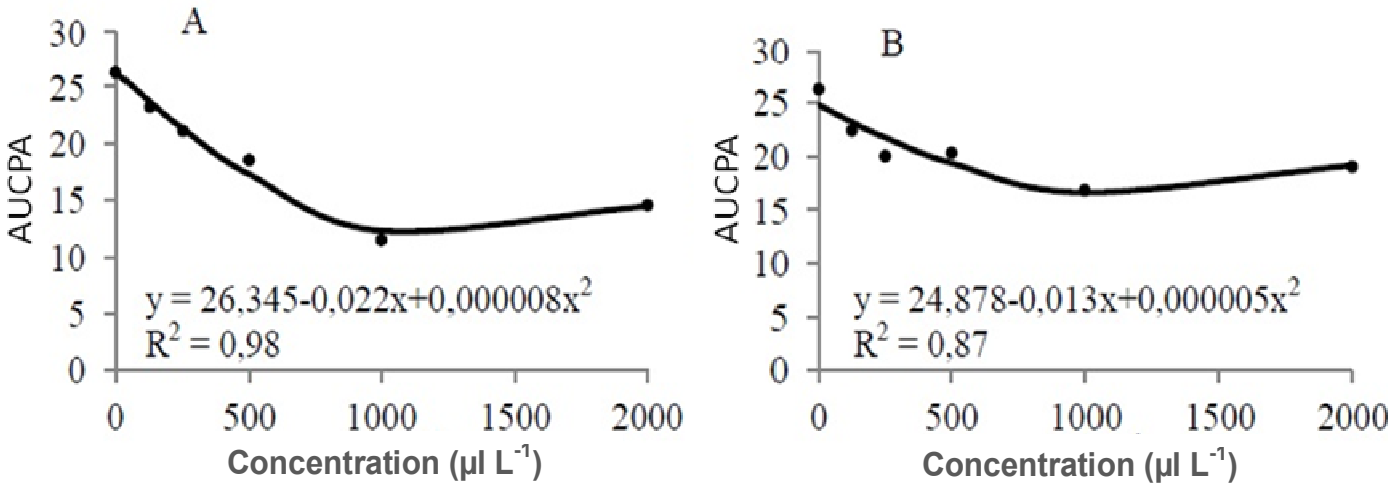

Figure 2. Area under the curve of progress of anthracnose (AUCPA) 35 days after inoculation of *C. lindemuthianum* in bean plants cultivar Pérola, treated with different concentrations (0, 125, 250, 500, 1000 and 2000 µL L^{-1}) of essential oils of *C. flexuosus* (A) and *V. polyanthes* (B). Data were transformed to √x + 0.5.

oils, showing specific reaction according to the chemical constitution of each oil.

Maia et al. (2010) evaluated essential oils of *Vernonia braziliana* and *Vernonia remotiflora* in Gram-negative and Gram-positive bacteria. The antimicrobial activity of these oils is related to the high amount of sesquiterpenes such as those found in this study: germacrene-D, bicyclo-germacrene, β-caryophyllene, and α-humulene (Table 1).

Montanari et al. (2011) attributed antimicrobial activity to sesquiterpene due to inhibition of breathing capacity and increased cell membrane permeability, as well as rupture of membrane integrity which results in leakage of K + ions and consequent loss of chemiosmosis control. Oils of *Lantana camara* and *Aloysia virgata*, rich in germacrene-D, showed moderate antibacterial activity against Gram-positive bacteria (*B. cereus* and *S. aureus*) and oil of *A. virgata* was active against Gram-negative bacteria (Costa et al., 2009).

Essential oil of *C. citratus* reduced the severity of anthracnose in passion fruit caused by *C. gloeosporioides*, with no significant difference in relation to Procloraz fungicide (Anaruna et al., 2010) or *Hibiscus rosa-sinensis* (Valencia et al., 2011).

Some authors attribute the partial control of other anthracnoses to citral. However, it is necessary to conduct experiments testing the isolated effect of neral and geranial. Garcia et al. (2008) reported that citral 1% was responsible for 70% control of anthracnose in papaya and 60% of anthracnose in banana. Essential oils rich in terpenes cause damage to lipids and proteins and break down cell walls and membranes, which results in cell lysis. In eukaryotic cells, essential oils destabilize the mitochondrial membrane and cause damage to plasma membrane proteins (Bakkali et al., 2008).

Direct effectiveness of phosphites against fungi is mainly related to phosphite ion, which seems to have a direct effect on the pathogen (Pereira et al., 2012). Furthermore, phosphites can also reduce sporulation of microorganisms, thereby causing reduction of pathogen inoculum potential (Silva et al., 2012).

The results of scanning electron microscopy in this study corroborate the results found in conidia germination *in vitro*, which had inhibition of germination with EOC, PP and EOV. Other authors have used SEM to demonstrate antifungal activity of essential oils (Pereira et al., 2011) treated leaves of coffee with *Cinnamomum zeylanicum* and *C. citratus* oils in control of *Cercospora coffeicola*. Through SEM observations, the authors found reduction in germination and mycelial size, with plasmolysis occurring in some conidia.

Induction of resistance in essential oils (EOs) may be related to their chemical constitution. It is known that EOs have various organic substances capable of inducing POX activity, such as terpene hydrocarbons, terpene alcohols, and simple alcohols, aldehydes, ketones, phenols, esters, ethers, oxides, peroxides, furans, organic acids, lactones, coumarins, and sulfur-containing compounds (Dewick, 2002). Thus, by chromatography data (Table 1), the presence of some of these substances which probably contributed to the increased levels of POX in bean tissues were confirmed. Pereira et al. (2012) found increased POX enzyme in coffee plants sprayed with essential oil of citronella, which resulted in decreased Cercospora leaf spot.

According to Daniel and Guest (2006), after treatment with phosphite there is accumulation of phenolic compounds in cells, formation of cytoplasmic aggregates and phenols around the infected cells and rapid increase in production of reactive oxygen species, followed by hypersensitivity reactions.

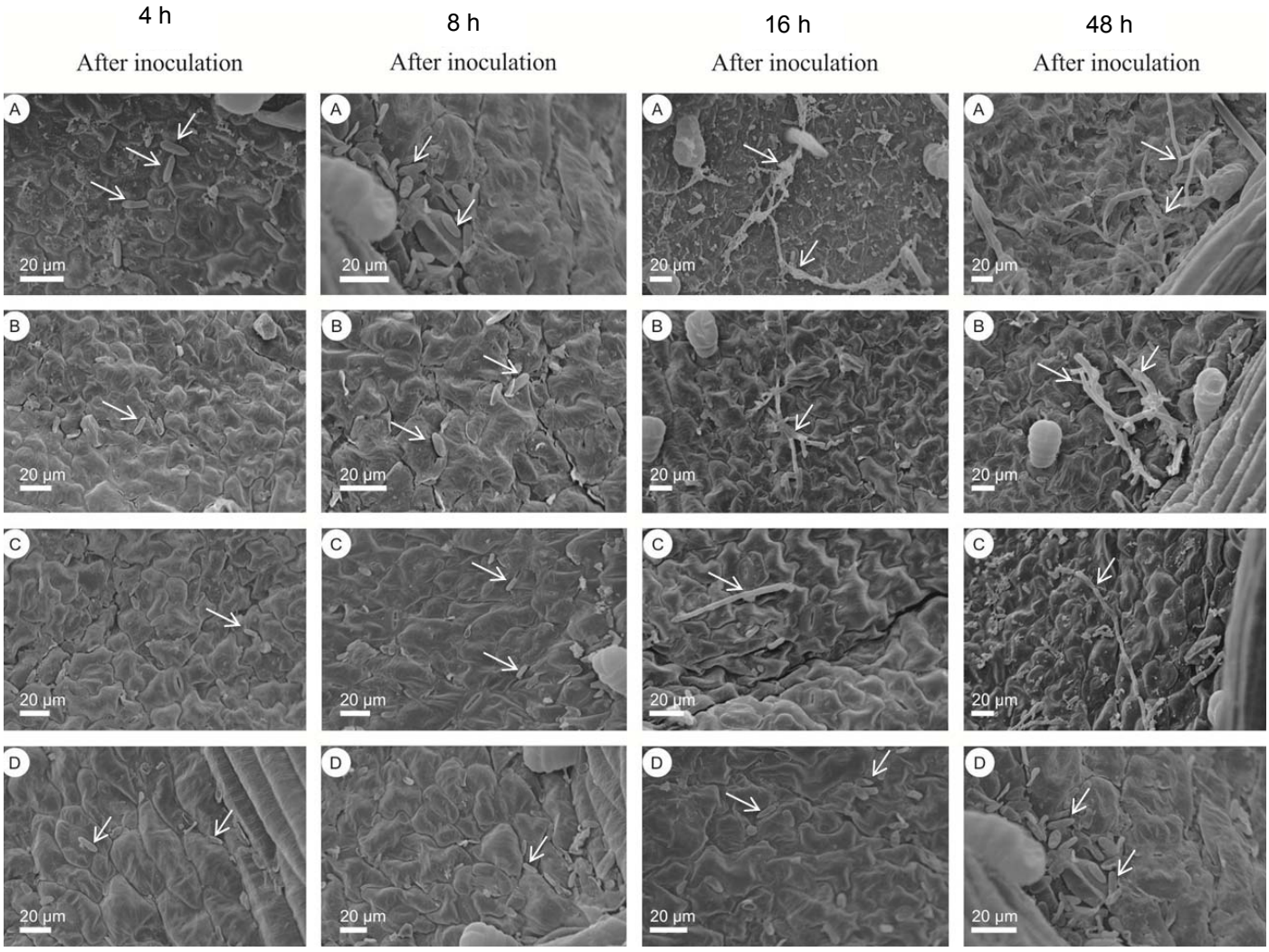

Figure 3. Scanning electron microscopy of bean leaves inoculated with *C. lindemuthianum* in different times after inoculation. Leaves were sprayed with distilled water (A), essential oil of *V. polyanthes* 1000 µl L^{-1} (B), *C. flexuosus* 1000 µl L^{-1} (C), and potassium phosphite 5 ml L^{-1} (D).

The positive relationship between POX activity of plant resistance to disease has been reported in several studies. Thus, increased POX activity could explain the lower severity of bean anthracnose found in plants treated with PP and EOC in severity experiments. Martins et al. (2013), also studying Pérola cultivar found increased activity of POX in plants treated with rhizobacteria and inoculated with *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. The authors also found decreased severity of disease at levels 42 to 76%.

Campos et al. (2004) reported that POX activity was significantly higher in bean plants treated with salicylic acid before and after inoculation of *C. lindemuthiaum*, with positive correlation between increased POX activity and anthracnose resistance. This resistance is related to POX ability to produce free radicals toxic to the pathogen in oxidative burst and to participate in lignin synthesis for strengthening cell wall. In addition, POX produces signal molecules such as H_2O_2, which can lead to expression of genes related to other resistance mechanisms (Hsu and Kao, 2003).

There was a significant difference regarding the activity of enzyme phenylalanine ammonia-lyase (PAL). Inoculated plants sprayed with PP had greater activity of PAL compared to plants sprayed with EOC and water at 8 and 10 days after spraying (Table 5). At day 13, plants sprayed with PP produced less PAL and did not differ from oil. This was possibly due to increase in PAL in early periods post inoculation (at 8 and 10 days after spraying), which caused a metabolic cost to the plant on the last evaluation day. This same effect occurred with plants treated with water, that is, PAL activity decreased considerably on the last day after inoculation (day 13). However, enzyme activity was lower than the activity of

elicitors PP and EOC, which resulted in increased severity of anthracnose in plants treated with water. Similar to PP treatment, inoculated plants sprayed with EOC showed higher PAL activity in all periods. This increase can mean that the entire phenylpropanoid pathway was altered, that is, mechanisms such as synthesis of lignin, phenolic compounds, quinones and others may have been potentialized by the products used in the experiment.

Non-inoculated plants showed no statistical difference between products at 8 and 13 days after spraying. Only at day 10, plants sprayed with PP had higher PAL activity. Thus, it is evident that PAL peak occurred at day 10. The highest PAL activity in plants treated with PP and essential oils has already been reported by other authors. Sellamuthu et al. (2013) found PAL to increase in avocado fruits with application of essential oil of thyme. Martins et al. (2013) reported increase in PAL levels in the second phenological stage of bean plants Pérola cultivar treated with growth promoting rhizobacteria. The authors also reported decreased severity of wilt of *C. flaccumfaciens* pv. *flaccumfaciens* in common bean by 76%, and increased dry weight of shoots and roots of treated plants. Campos et al. (2003) reported that bean plants Pérola cultivar treated with salicylic acid had higher PAL activity than plants treated with water, and plants challenged with the pathogen *C. lindemuthiahum* showed higher PAL activity than non-inoculated plants.

PAL catalyzes the deamination reaction of L-phenylalanine to trans-Cinnamic acid. This process is the first step in the phenylpropanoid pathway which is highly important for the production of plant defense compounds against pathogens (Mandal et al., 2009).

Thus, essential oil of *C. flexuosus* and potassium phosphite could be an alternative for the management of bean anthracnose, because in addition to significant induction of defense enzymes such as POX and PAL, these products also presented antifungal proprieties. However, more studies need to be performed to assess the efficiency of these products in anthracnose control in field conditions, doses to be used and toxicology to man and the environment, as well as the adequacy of essential oil production and oil formulation.

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Conflict of interests

The author(s) have not declared any conflict of interests.

REFERENCES

Adukwu EC, Allen SCH, Phuillips CAA (2012). The anti-biofilm activity of essential oils of lemon grass *Cymbopogon flexuosus* and grapefruit *Citrus paradisi* about five isolates of *Staphylococcus aureus*. J. Appl. Microbiol. 113:1217-1227.

Alzate DAOG, Afanador IMM, Durango LK, Garcia DLR (2009). Evaluation of phytotoxicity and Antifungal activity against *Colletotrichum acutatum* of essential oils of thyme (*Thymus vulgaris*), timoncillo (*Mymbopogon citratus*), and its major components. Rev. Fac. Quím. Pharm. 16:116-124.

Anaruma ND, Schmidt FL, Duarte MCT, Figueira GM, Delarmelina C, Benato EA, Sartoratto A (2010). Control of *Colletotrichum gloeosporioides* (Penz.) Sacc. in yellow passion fruit using *Cymbopogon citratus* essential oil. Braz. J. Microbiol. 41(1):66-73.

Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008). Biological effects of essential oils-a review. Food Chem. Toxicol. 46(2):446-475.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72(1):248-254.

Campa A, Giraldez R, Ferreira JJ (2011). Genetic analysis of the resistance to eight anthracnose races in the common bean differential cultivar Kaboon. Phytopathology 101(6):757-764.

Campos ÂD, Ferreira AG, Hampe MMV, Antunes IF, Brancão N, Silveira EP, Silva JBd, Osório VA (2003). Induction of chalcone synthase and phenylalanine ammonia-lyase by salicylic acid and *Colletotrichum lindemuthianum* in common bean. Braz. J. Plant Physiol. 15(3):129-134.

Campos, A, Ferreira, AG, Hampe, MMV, Antunes, IF, Brancão, N, Silveira, Ed, Osório, VA, Augustin, E (2004). Peroxidase and polyphenol oxidase activity in bean anthracnose resistance. Pesqui. Agropecu. Braz. 39(7):637-643.

Chance B, Maehly A (1955). Assay of catalases and peroxidases. Methods Enzymol 2:764-775.

Costa EV, Pinheiro MLB, Silva JRdA, Maia BHLdN, Duarte MCT, Amaral ACF, Machado GMdC, Leon LL (2009). Antimicrobial and antileishmanial activity of essential oil from the leaves of *Annona foetida* (Annonaceae). Quím. Nova 32(1):78-81.

Damasceno SK, De Souza E, Ishikawa F (2007). Characterization of *Colletotrichum lindemuthianum* isolates from the state of Minas Gerais, Brazil. J. Phytopathol. 155(4):241-247.

Daniel R, Guest D (2006). Defence responses induced by potassium phosphonate in *Phytophthora palmivora* challenged *Arabidopsis thaliana*. Physiol. Mole. Plant Pathol. 67(3):194-201.

Dewick PM (2002). Medicinal Natural Products: A Biosynthetic Approach, 3rd Edition. John Wiley & Sons.

Garcia R, Alves ES, Santos MP, Aquije GM, Fernandes AAR, Santos RBd, Ventura JA, Fernandes P (2008). Antimicrobial activity and potential use of monoterpenes as tropical fruits preservatives. Braz. J. Microbiol. 39(1):163-168.

Gillard C, Ranatunga N, Conner R (2012). The effect of foliar fungicide application timing on the control of dry bean anthracnose. Can. J. Plant Sci. 92(1):109-118.

Godoy CV, Koga LJ, Canteri MG (2006). Diagrammatic scale for assessment of soybean rust severity. Phytopathol. Braz. 31(1):63-68.

Hsu SY, Kao CH (2003). Differential effect of sorbitol and polyethylene glycol on antioxidant enzymes in rice leaves. Plant Growth Regul. 39(1):83-90.

Ishikawa FH, de Souza, EA, Davide, LM (2008). Genetic variability within isolates of *Colletotrichum lindemuthianum* belonging to race 65 from the state of Minas Gerais, Brazil. Biologia 63(2):156-161.

Ishikawa FH, Souza EA, Shoji JY, Connolly L, Freitag M, Read ND, Roca MG (2012). Heterokaryon incompatibility is suppressed following conidial anastomosis tube fusion in a fungal plant pathogen. PloS one 7(2):e31175.

Kar M, Mishra D (1976). Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiol. 57(2):315-319.

Maia AIV, Torres MCM, Pessoa ODL, Menezes J, Costa SMO, Nogueira VLR, Melo VMM, Souza E, Cavalcante MGB, Albuquerque M (2010). Volatile leaf oils of *Vernonia Brasiliana* and *Vernonia Remotiflora*: chemical composition and biological activity. Quim. Nova 33:584-586.

Mandal S, Mallick N, Mitra A (2009). Salicylic acid-induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato. Plant Physiol. Biochem. 47(7):642-649.

Martins SJ, de Medeiros FHV, de Souza RM, de Resende MLV, Ribeiro Junior PM (2013). Biological control of bacterial wilt of common bean by plant growth-promoting rhizobacteria. Biol. Control 66(1):65-71.

Montanari RM, Barbosa LC, Demuner AJ, Silva CJ, Carvalho LS, Andrade NJ (2011). Chemical composition and antibacterial activity of essential oils from Verbenaceae species: Alternative sources of (E)-caryophyllene and germacrene-D. Quím. Nova 34(9):1550-1555.

Pereira RB, Lucas GC, Perina FJ, Resende MLVd, Alves E (2011). Potential of essential oils for the control of brown eye spot in coffee plants. Ciênc. Agrotechol. 35(1):115-123.

Pereira VF, de Resende MLV, Júnior PMR, de Albuquerque Regina M, da Mota RV, Vitorino LRR (2012). Potassium phosphite on the control of downy mildew of grapevine and physicochemical characteristics of Merlot grapes. Pesq. Agropec. Braz. 47(11):1581-1588.

Rasooli I, Rezaei MB, Allameh A (2006). Growth inhibition and morphological alterations of *Aspergillus niger* by essential oils from *Thymus eriocalyx* and *Thymus x-porlock*. Food Control 17(5):359-364.

Rosado LDS, Pinto JEBP, Bertolucci SKV, Jesus HCRd, Alves PB (2013). Changes in the content and composition of the essential oil of *Ocimum basilicum* L. during storage. J. Essential Oil Res. 25(3):227-232.

Sellamuthu PS, Sivakumar D, Soundy P, Korsten L (2013). Essential oil vapours suppress the development of anthracnose and enhance defence related and antioxidant enzyme activities in avocado fruit. Postharvest Biol. Technol. 81:66-72.

Shaner G, Finney R (1977). The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67(8):1051-1056.

Valencia LD, Castro SD, Pascual CB, Magdalita PM (2011). Lemongrass *Cymbopogon citratus* (DC.) Stapf. oil: potential biocontrol agent against major fungal pathogens of gumamela *Hibiscus rosasinensis* L. Philipp. J. Crop Sci. 36(3):70-75.

Van den Dool H, Dec Kratz P (1963). A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. J. Chromatogr. A 11:463-471.

Zucker M (1965). Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiol. 40(5):779-784.