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RESEARCH ARTICLE / *ARTIGO*

Effectiveness of essential oils in the treatment of *Colletotrichum truncatum*-infected soybean seeds

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ABSTRACT

The aim of the study was to evaluate the antifungal activity of essential oils from "hortelã do campo" (*Hyptis marrubioides*), "alfazema-do-Brasil" (*Aloysia gratissima*) and "erva-baleeira" (*Cordia verbenacea*) and their efficacy in the treatment of soybean seeds infected with *Colletotrichum truncatum*. *In vitro* assays were performed to evaluate the effects of the oils on spore germination, mycelial growth, and the production and viability of *C. truncatum* conidia. Soybean seeds inoculated with *C. truncatum* were treated with essential oils at concentrations ranging from 0.5 to 2% and grown under greenhouse conditions. Seed health and germination percentages were evaluated according to standard protocols. Seedling emergence, stand establishment and the percentages of dead seedlings were determined, together with the emergence speed index, plantlet height and dry weight of aerial biomass. The oils from *H. marrubioides*, *C. verbenacea* and *A. gratissima* inhibited the germination and production of conidia as well as the growth of *C. truncatum*. At concentrations ≥ 1%, the effectiveness of the oils against soybean anthracnose was superior to, or at least comparable with, that of the fungicide carbendazim. The viability of conidia and the germination of soybean seeds were not affected by any of the treatments with essential oils. It is concluded that essential oils from *H. marrubioides*, *A. gratissima* and *C. verbenacea* have potential as alternatives to synthetic fungicides in the control of anthracnose in soybean seeds.

Key words: *Glycine max*, alternative control, anthracnose, seed treatment.

INTRODUCTION

Most fungi that are pathogenic to soybean (*Glycine max*) are transmitted to new areas through infected seeds, a practice that can cause considerable damage to the crop and significant reductions in productivity (Hamawaki et al., 2002). Anthracnose caused by *Colletotrichum truncatum* is one of the most common and damaging seed-dispersed pathogens to affect soybeans, particularly when the crop is grown under hot and humid conditions. This pathogen can attack all plant parts at every developmental stage and induces symptoms that include the collapse of seedlings, necrosis of petioles and veins, appearance of yellow to brown spots on leaves and stems, and rotting of roots and pods (Galli et al., 2007). Additionally, the presence of pathogens in seeds may lead to significant reductions in seed germination, plant emergence and vigor, duration of seed storage and crop yield (Ito & Tanaka, 1993).

The most straightforward method of avoiding the dissemination of seed-dispersed pathogens is the use of certified batches of healthy seeds. Nevertheless, treatment of batches of potentially infected seeds with biocides prior to cultivation is more economical, reasonably efficient and commonly employed to control the dissemination of phytopathogens in the field (Machado, 2000). Various natural plant products are known to be effective against seed-associated pathogens (Souza et al., 2002; Atanda et al., 2007; Brand et al., 2007; Slusarenko et al., 2008; Dhingra et al., 2009; Koch et al., 2010; Steffen et al., 2010), while the essential oils from the traditional Brazilian medicinal plants "hortelã do campo" [*Hyptis marrubioides* (Lamiaceae)], "alfazema-do-Brasil" [*Aloysia gratissima* (Verbenaceae)] and "erva-baleeira" [*Cordia verbenacea* (Boraginaceae)] have been shown to be effective in the control of Asian soybean rust (Silva et al., 2012a,b). In this context, we have investigated the effect of essential oils from these medicinal plant species on *C. truncatum*-infected seeds and, ultimately, on the control of anthracnose under greenhouse conditions.

MATERIALS AND METHODS

Source of *C. truncatum*

Colletotrichum truncatum was obtained from contaminated soybean seeds, and monosporic cultures were prepared according to Silva et al. (2009). The isolate (reference CML1899) was deposited in the Mycological Collection of the Plant Pathology Department at Lavras Federal University (UFLA), Lavras, MG, Brazil.

Extraction and analysis of essential oils

Plants of *H. marrubioides*, *A. gratissima* and *C. verbenacea* were cultivated in the medicinal garden within the campus at UFLA, and voucher specimens were deposited in the University herbarium with reference numbers 1022, 19810, and 7982, respectively. Aerial parts of the plants were collected in the morning during June 2008, and immediately ground and transferred to a modified Clevenger apparatus. Essential oils were collected after 2 h of hydrodistillation and stored at - 40ºC in aluminum foil-covered glass vials until required for use.

Oil samples were subjected to analysis by gas chromatography (GC) with flame ionization detection using a Shimadzu (Kyoto, Japan) model 17A chromatograph, and to GC-mass spectrometry (MS) using a Shimadzu model QP5050A instrument with a quadrupole detector. In each case, a 5% phenyl methylpolysiloxane fused-silica capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) was employed. The chromatographic conditions were: oven temperature – initially at 50ºC, increased to 200ºC at 4ºC/min, held at 200ºC for 5 min, and finally increased to 280ºC at 20ºC/min; carrier gas – helium at a flow rate of 1.2 mL/min; injector temperature – 220ºC; injection volume – 0.5 µL; flow ratio – 1:20; interface temperature – 240 ºC; ionization potential – 70 eV; mass range – 40-550 *m/z*; scan rate - 0.84 scans/s. The compounds were identified by comparison of their MS with those available at NIST (1998) on-line library and in a literature database (Adams, 2007). Kovats retention indices (KI) were determined from a calibration curve prepared with a series of *n*-alkanes (C_8-C_{32}) chromatographed under conditions identical to those employed for sample analysis, and compared with values reported in the literature (Adams, 2007). The relative concentrations of the essential oil components were calculated using the area normalization method without considering specific response factors.

Effects of essential oils on the germination, growth and development of *C. truncatum in vitro*

The essential oils were mixed separately with 1% Tween 20 in water to produce concentrated emulsions. Aliquots of 1 mL were combined with 9 mL of autoclaved water-agar medium maintained just above the melting point and poured immediately into 9-cm diameter Petri dishes. The final concentrations of oils in the media were 0.1, 0.3, 0.5, 1 or 2%. Medium for the negative control was prepared in exactly the same manner but omitting the essential oils. Aliquots of 50 µL of a *C. truncatum* conidial suspension containing 2 x 10^4 spores/mL were spread evenly onto the solidified media and incubated at 25ºC under light conditions for 9 h. The numbers of germinated conidia (defined as those in which the size of the germ tube was equal to, or greater than, the spore diameter) were counted in samples of 200 conidia within each quadrant of the Petri dishes, and the values compared with those obtained with the negative control.

In order to assess mycelial growth, Petri dishes containing potato dextrose agar (PDA) supplemented with essential oils (0.1, 0.3, 0.5, 1 or 2%) were prepared as described above. The positive control medium contained the broad-spectrum fungicide carbendazim (Derosal 500 SC; 1.25 g/plate), while the negative control medium was supplemented only with aqueous 1% Tween 20. Discs of 8 mm in diameter obtained from the edges of 10-day old fungal colonies, were placed, one per Petri dish, on top and in the centre of the medium and the dishes were incubated at 25ºC under a 12:12 h photoperiod for 9 days. Two orthogonal measurements of colony diameter were performed at a fixed time each day from the 2nd to the 9th day. The mycelial growth index (MGI; mm/day) was calculated from the expression $MGI = \Sigma(D - Da) / N$, in which D is the actual mean colony diameter, Da is the previous mean diameter, and N is the number of days of growth (Araújo et al., 2008).

The production of conidia was assessed by counting the number of spores in each Petri dish with the aid of a hemocytometer according to the method of Ribeiro & Bedendo (1999). In order to determine viability, aliquots of 100 µL of conidial suspensions were spread onto water-agar medium and incubated at 25°C under light conditions for 9 h. Trypan blue stain in lactoglycerol was used to suppress conidial germination and to determine the number of dead conidia.

All assays were conducted in a randomized design with four replications, each plate representing one repetition.

Inoculation of soybean seeds with *C. truncatum*

PDA medium with an osmotic potential of -0.7 MPa was prepared by the addition of mannitol (Michel & Radcliffe, 1995), and 10 mL portions of the sterilized medium were transferred to 9 cm Petri dishes and allowed to solidify. Aliquots of 300 µL of a *C. truncatum* conidial suspension containing 10^6 spores/mL were spread evenly onto the solidified medium and incubated at 25ºC under a 12:12 h photoperiod. After 8 days of incubation, pathogen-free soybean seeds cultivar MGBR-46 were spread on the surface of the medium in the form of a single layer in order to ensure close contact with the fungal colony. Incubation was continued under the same conditions for a further 40 h (Galli et al., 2005), after which the seeds were removed and dried in a laminar flow cabinet for 24h.

Effects of essential oils on seed health and germination

Seeds inoculated with *C. truncatum* were treated separately with essential oils from *H. marrubioides*, *A. gratissima* or *C. verbenacea*, each at concentrations of 0.5, 1 or 2%, or with the fungicide carbendazim (50 g a.i./100 kg of seeds). Inoculated seeds were immersed for 2 min in solutions of the appropriate essential oil or fungicide, air-dried in a laminar flow cabinet for 24 h and submitted to the blotter test described in the manual issued by MAPA (2009). Seeds were subsequently maintained at 20ºC under a 12:12 h photoperiod for eight days and then examined

individually under the stereomicroscope to determine the level of *C. truncatum* infection. Inoculated non-treated seeds were employed in the negative control. The whole experiment was conducted according to a randomized design with 11 treatments repeated four times with 50 seeds per replicate totaling 200 seeds per treatment.

The standard germination test (roll paper method) described in the manual issued by MAPA (2009) was employed to determine the percentages of normal, abnormal and dead seedlings that emerged from inoculated treated seeds, inoculated non-treated seeds and non-inoculated non-treated seeds.

Effects of essential oils on emergence and development of soybean seedlings under greenhouse conditions

Seeds inoculated with *C. truncatum* were treated with essential oils or fungicide (as described above), while controls were inoculated non-treated seeds and non-inoculated non-treated seeds. Seeds were sown in plastic trays (8 L capacity) containing commercial substrate Plantmax DDL Agro Indústria®, previously autoclaved for 1 h at 121ºC and 1 atm, and were irrigated daily by means of a controlled micro-spray system. The emergence speed index (ESI) was determined according to the methodology described by Maguire (1962). Evaluations were conducted daily by counting the number of seedlings that emerged until the stabilization of the plant population. On the 14th day after sowing, stand establishment and the percentages of dead and germinated seedlings were assessed. On the 30th day after sowing, each plantlet was cut at the soil surface and its height recorded. In order to determine dry weight biomass, plantlets were transferred to paper bags and maintained in a forced air oven at 60ºC until constant weight was attained. The whole experiment was conducted according to a randomized block design with 12 treatments replicated four times with 50 seeds per replicate totaling 200 seeds per treatment.

Statistical analyses

The experiments were conducted in duplicate. Data were subjected to analysis of variance (ANOVA): normality and homogeneity of variance were evaluated by inspection of the residual plots and no deviations from the assumptions were observed. Scott Knott tests ($\alpha = 0.05$) were applied, where appropriate, in order to determine the significance of the differences between mean values. Quantitative variables were submitted to regression analysis.

RESULTS

Compositions of essential oils

Chromatographic analysis revealed a considerable diversity in the composition and in the proportions of the various chemical classes present in essential oils derived from *A. gratissima, C. verbenacea* and *H. marrubioides* (Table 1). The oil from *A. gratissima* presented the largest number of components (39), followed by *C. verbenacea* oil (26) and *H. marrubioides* oil (24). β-Pinene, *trans*-pinocamphone and *trans*-pinocarvyl acetate were the main constituents of *A. gratissima* oil. Methyl (2*E*,6*E*)-farnesoate and β-caryophyllene were the principal components of *C. verbenacea* oil, while the major constituents of *H. marrubioides* oil were *cis*- and *trans*-thujone.

Effects of essential oils on seed germination by the roll paper method

Germination tests revealed that 83.5% of the seedlings that emerged from non-inoculated non-treated seeds were free from fungal infection and, of these, 65.5% showed normal development, 16.5% were abnormal and 1.5% died. Infection by a variety of fungal species, especially *Penicillium* sp. and *Aspergillus* sp., was detected in the remaining seedlings (16.5%) that developed from non-inoculated non-treated seeds, and all were classified as abnormal (Table 2). Treatment with essential oils had no negative effect on seed germination. All seedlings that emerged from *C. truncatum*-inoculated soybean seeds were infected, irrespective of the treatment applied. Typically, more than 90% of these seedlings exhibited abnormal development and up to 9.5% died because of infection. These results reveal the detrimental effects caused by *C. truncatum* infection in seeds.

Effects of essential oils on seed health

The seed health test revealed that treatments with essential oils at all concentrations, except for *A. gratissima* oil at 0.5%, provided significant reductions in the incidence of *C. truncatum* infection in inoculated soybean seeds compared with the inoculated non-treated controls (Table 2). Indeed, the essential oils were generally more effective than the fungicide in controlling *C. truncatum* infection in inoculated seeds, although the efficacy of treatments with *H. marrubioides* oil at 1%, or *C. verbenacea* oil at 0.5%, were similar to that of the fungicide. The lowest incidence of the pathogen was recorded with *C. verbenacea* oil at 2%.

Effects of essential oils on emergence and development of soybean seedlings under greenhouse conditions

The highest percentages of seedlings emerged from inoculated seeds treated with oils from *H. marrubioides* at 2%, from *A. gratissima* at 0.5, 1 or 2%, or from *C. verbenacea* at 1 or 2% (Table 2). These percentages were significantly larger than those recorded for non-inoculated non-treated seeds and for inoculated seeds that received no treatment or were treated with fungicide.

The highest stands were observed for seedlings derived from inoculated seeds treated with *H. marrubioides* or *A. gratissima* oils at 2%, or with *C. verbenacea* oil at 1 or 2% (Table 2). The treatment of inoculated seeds with fungicide resulted in stands similar to those obtained following treatment with *H. marrubioides* or *C. verbenacea* oils at 0.5%.

TABLE 1 - Relative percentages of the constituents of essential oils derived from *Aloysia gratissima*, *Cordia verbenacea* and *Hyptis marrubioides* as determined by GC/MS analysis

Retention index	Compound	Relative area (%)		
		Aloysia gratissima	*Cordia verbenacea*	*Hyptis marrubioides*
863	(Z) Salvene	-[a]	-	0.61
924	α-Thujone	0.34	-	-
931	α-Pinene	3.23	0.74	-
947	Canfene	0.20	-	-
970	Sabinene	1.07	0.81	9.62
975	β-Pinene	27.05	-	1.01
988	Myrcene	3.82	-	-
1023	p-Cimene	0.26	-	-
1028	Limonene	3,67	3.59	-
1029	β-phelandrene	0.33	2.10	-
1030	1,8-Cineol	0.26	0.62	1.68
1045	(E)-β-Ocymene	0.85	-	-
1100	Linalool	0.57	-	0.47
1106	*cis*-Thujone	-	-	43.49
1117	*trans*-Thujone	-	-	15.18
1126	α-Canphonelal	0.30	-	-
1137	*iso*-3-Thujanol	-	-	0.51
1139	*trans*-Pinocarveol	2.02	-	-
1145	*trans*-Verbenol	1.14	-	1.22
1160	*trans*-Pinocanphone	11.93	-	-
1161	Pinocarvone	1.04	-	-
1175	*cis*-Pinocamphone	3.95	-	4.34
1179	Terpinen-4-ol	0.26	-	0,59
1194	Myrtenal	1.64	-	0.70
1283	bornylacetate	0.46	-	-
1294	*trans*-pinocarvyl acetate	8.94	-	-
1374	α-Copaene	-	0.29	3.48
1382	β-Bourbonene	0.31	-	0.68
1388	β-Elemene	0.31	0.42	-
1418	β-Caryophyllene	2.81	26,2	5.07
1427	γ-Elemene	*	-	-
1431	α-*trans*-Bergamotene	-	0.36	-
1447	Geranyl acetone	-	*	-
1454	α-Humulene	0.82	7.60	0.39
1479	Germacrene D	2.71	1.45	2.87
1493	Bicyclogermacrene	1.97	2.99	-
1505	Germacrene A	*	-	-
1513	γ-Cadinene	-	-	0.86
1515	Cubebol	0.48	-	-
1546	Elemol	0.36	-	-
1548	Unidentified	-	-	0.38
1557	Germacrene B	2.85	-	-
1560	Unidentified	-	-	0.79
1575	Spathulenol	1.30	1.03	-
1580	Caryophillene oxide	1.87	3.01	2.17
1595	Guaiol	6.23	-	-
1608	Epóxi II Humulene	-	0.54	-
1612	Rosifoliol	0.66	-	-
1619	Epi-α-Cadinol	0.24	1.2	-
1632	Caryophylla-4(12),8(13)-dien-5 α-ol	-	-	0.53
1635	Caryophylla-4(12),8(13)-dien-5 β-ol	-	-	1.27
1645	α-Muurolol	0.32	-	-
1654	Unidentified	0.31	-	-
1655	Unidentified	-	0.42	-
1662	ar-Turmerone	-	1.45	-
1663	Bulnesol	2.11	-	-
1667	Turmerone	-	2.10	-
1683	Eudesma-4(15),7-dien-1 β-ol	-	-	1.59
1685	Unidentified	-	2.15	-
1699	Curlone	-	0.52	-
1707	Methyl 3,7,11-trimethyl 6,10-Dodecadieneate	-	0.51	-
1713	2*E*,6*Z*-Pharnesol	-	0.55	-
1735	2*E*,6*E*-Pharnesal	-	0.31	-
1778	Methyl 2*E*,6*E*-Pharnesoate	-	35.85	
1870	Methyl 2*E*,6*E*-Pharnesoate epoxide	-	2.81	-

[a]Not detected; *Not quantified.

TABLE 2 - Emergence and development tests determined under greenhouse conditions, seed germination test done with the roll paper method and soybean seed health in the laboratory. Soybean seeds were artificially inoculated with *Colletotrichum truncatum* and treated with essential oils

Treatment (oil concentration)	Incidence of infection (%)	Emerged seedlings (%)	Stand establishment (%)	Dead seedlings (%)	Emergence speed index	Dry biomass (g)	Plant height (cm)	Seed germination test by roll paper method	
								Dead (%)	Abnormal (%)
Inoculated seeds treated with *Hyptis marrubioides* oil									
0.5%	57.5 ^{d}	52.0 ^{a}	25.0 ^{b}	27.0 ^{c}	6.9 ^{a}	6.0 ^{a}	24.8 ^{b}	5.5 ^{b}	94.5 ^{b}
1.0%	48.5 ^{c}	62.0 ^{a}	48.0 ^{c}	14.0 ^{b}	9.0 ^{a}	11.4 ^{b}	24.5 ^{b}	5.5 ^{b}	94.5 ^{b}
2.0%	35.5 ^{b}	72.5 ^{b}	63.5 ^{d}	9.0 ^{a}	10.6 ^{b}	18.7 ^{c}	32.9 ^{c}	5.0 ^{b}	95.0 ^{b}
Inoculated seeds treated with *Aloysia gratissima* oil									
0.5%	72.0 ^{e}	71.5 ^{b}	44.5 ^{c}	27.0 ^{c}	10.9 ^{b}	11.1 ^{b}	30.3 ^{c}	9.5 ^{b}	91.0 ^{b}
1.0%	39.5 ^{b}	72.5 ^{b}	54.0 ^{c}	18.50 ^{b}	11.0 ^{b}	15.4 ^{c}	31.6 ^{c}	5.0 ^{b}	95.0 ^{b}
2.0%	38.5 ^{b}	85.0 ^{b}	74.5 ^{d}	10.5 ^{a}	13.5 ^{b}	16.6 ^{c}	33.4 ^{c}	5.5 ^{b}	94.5 ^{b}
Inoculated seeds treated with *Cordia verbenacea* oil									
0.5%	48.5 ^{c}	64.0 ^{a}	25.5 ^{b}	38.5 ^{d}	9.7 ^{a}	5.8 ^{a}	23.9 ^{b}	6.0 ^{b}	94.0 ^{b}
1.0%	33.5 ^{b}	80.5 ^{b}	63.0 ^{d}	17.5 ^{b}	12.5 ^{b}	13.5 ^{b}	33.1 ^{c}	8.0 ^{b}	92.0 ^{b}
2.0%	20.5 ^{a}	78.5 ^{b}	61.5 ^{d}	17.0 ^{b}	12.4 ^{b}	13.4 ^{b}	32.8 ^{c}	9.5 ^{b}	90.5 ^{b}
Inoculated non-treated seeds	79.5 ^{e}	54.0 ^{a}	11.5 ^{a}	42.5 ^{d}	9.0 ^{a}	4.4 ^{a}	20.3 ^{a}	6.0 ^{b}	94.0 ^{b}
Non-inoculated non-treated seeds	-	59.5 ^{a}	54.5 ^{c}	5.0 ^{a}	9.5 ^{a}	18.0 ^{c}	35.7 ^{c}	0 ^{a}	16.5 ^{a}
Inoculated seeds treated with fungicide	52.0 ^{c}	57.0 ^{a}	37.5 ^{b}	19.5 ^{b}	8.5 ^{a}	11.1 ^{b}	32.2 ^{c}	4.0 ^{b}	96.0 ^{b}
CV (%)	22.6	14.9	20.5	25.3	19.5	19.6	7.7	35.3	3.9

In each column, values followed by different superscript lowercase letters are significantly different (Scott Knott test; $\alpha = 0.05$). - Not determined.

At the 14th day after sowing, 42.5% of the seedlings that emerged from inoculated non-treated seeds, and 38.5% of the seedlings that emerged from inoculated seeds treated with *C. verbenacea* oil at 0.5%, died because of damping-off (Table 2). For most other essential oil treatments, and for the treatment involving commercial fungicide, the percentages of dead seedlings were significantly lower than the values reported above, but were significantly higher than the 5% recorded with non-inoculated non-treated seeds. However, treatment with *H. marrubioides* or *A. gratissima* oils at 2% gave percentages of dead seedlings that were not significantly different from those obtained with non-inoculated non-treated seeds, thus demonstrating the effectiveness of these oils in controlling *C. truncatum* infection. Indeed, at concentrations of 1%, the efficacies of oils from *H. marrubioides* and *A. gratissima* in controlling anthracnose were similar to that of the fungicide, while at concentrations of 2% the efficacies of these essential oils were significantly higher ($P \leq 0.05$) than that of the fungicide.

The highest ESI values were recorded for seedlings that emerged from inoculated seeds treated with oils from *H. marrubioides* at 2%, from *A. gratissima* at 0.5, 1 or 2%, or from *C. verbenacea* at 1 or 2% (Table 2). On the other hand, the ESI values recorded for inoculated seeds treated with fungicide, or with oil from *H. marrubioides* at 0.5 or 1%, or oil from *C. verbenacea* at 0.5%, were low and not significantly different from those determined for inoculated non-treated seeds and non-inoculated non-treated seeds.

Seedlings that emerged from non-inoculated non-treated seeds, or from inoculated seeds treated with oil from *H. marrubioides* at 2%, or oil from *A. gratissima* at 1 or 2%, presented the highest values of dry biomass (Table 2). Inoculated seeds treated with *H. marrubioides* or *C. verbenacea* oils at 0.5% yielded seedlings with the lowest biomass values, although these were not significantly different from those of plantlets that emerged from inoculated non-treated seeds.

The tallest seedlings were derived from non-inoculated non-treated seeds or from inoculated seeds treated with fungicide or with essential oils at 1 or 2%, although such seedlings were not significantly different in size (Table 2). The smallest plants were those that emerged from inoculated non-treated seeds.

Effects of essential oils on the germination, growth and development of *C. truncatum in vitro*

The germination of *C. truncatum* conidia in negative control medium supplemented with aqueous 1% Tween 20 was 95.5%. Addition of emulsions of essential oils in aqueous 1% Tween 20 to the medium gave rise to 100% inhibition of conidial germination at all concentrations assayed (0.1 to 2%) with the single exception of oil from *C. verbenacea* which, at the 0.1% level, resulted in 25% inhibition of germination (data not shown).

The growth of *C. truncatum* mycelium was also inhibited by the presence of essential oils in the medium, although the levels of inhibition varied according to the source of the oil and its concentration. Supplementation with essential oils from *A. gratissima* or *H. marrubioides* at levels of 0.5, 1 or 2% greatly reduced fungal growth (Figure 1). These oils showed, respectively, 99.2 and 94.7% inhibition of mycelial growth at the highest oil concentrations assayed. In contrast, the essential oil from *C. verbenacea* was less efficient in reducing the growth of mycelium (Figure 1), while the commercial fungicide completely inhibited fungal growth (Data not shown).

Conidial production decreased with increasing concentrations of the essential oils from *H. marrubioides* and *A. gratissima* (Figure 1) while, in the presence of *C. verbenacea* oil, production was low even at the lowest oil concentration (0.1%). There were no significant differences between the inhibitory effects on conidia production of the three essential oils when present at concentrations higher than 0.5%. The viabilities of conidia produced in the presence of the essential oils and on negative control plates were not significantly different (data not shown).

DISCUSSION

A number of reports have described the effects of plant extracts and essential oils on phytopathogens, but only a few have focused on the antifungal potential of the oils from *H. marrubioides*, *A. gratissima* and *C. verbenacea*.

The present study is the first to consider the potential use of the essential oils from *H. marrubioides*, *A. gratissima* and *C. verbenacea* in the control of *C. truncatum*, although the effectiveness of oils from other plant species in controlling anthracnose has been addressed (Silva et al., 2009). Essential oils from *H. marrubioides, A. gratissima* and *C. verbenacea* have been shown to completely inhibit the germination of *Phakopsora pachyrhizi* urediniospores even at low concentrations ($\geq 0.05\%$), and are reportedly effective in controlling Asian soybean rust in greenhouse experiments (Silva et al., 2012a,b). The complete inhibition of conidial germination in *Botrytis cinerea, C. truncatum* and *Fusarium oxysporum* by extracts of *Caryocar brasiliense* at concentrations greater than 100 mg/L has also been described (Marques et al., 2002).

Silva et al. (2009) demonstrated that the growth of *C. gloeosporioides* mycelium was completely inhibited by essential oils from *C. citratus, L. citriodora, L. sidoides, O. gratissimum* and *Rosmarinus officinalis,* but not by an aqueous extract of *C. verbenacea.* However, results from the present study have revealed that the essential oil from the latter specie was able to reduce the growth of *C. truncatum*, although it was not as effective as the oil from *H. marrubioides* or *A. gratissima*. The difference in antifungal activity between the aqueous extract and the essential oil of the same species may be explained in terms of differential composition (Da Silva, 2006). The main classes of compounds present in essential oils are phenols, terpenes and aldehydes (Ceylan & Fung, 2004),

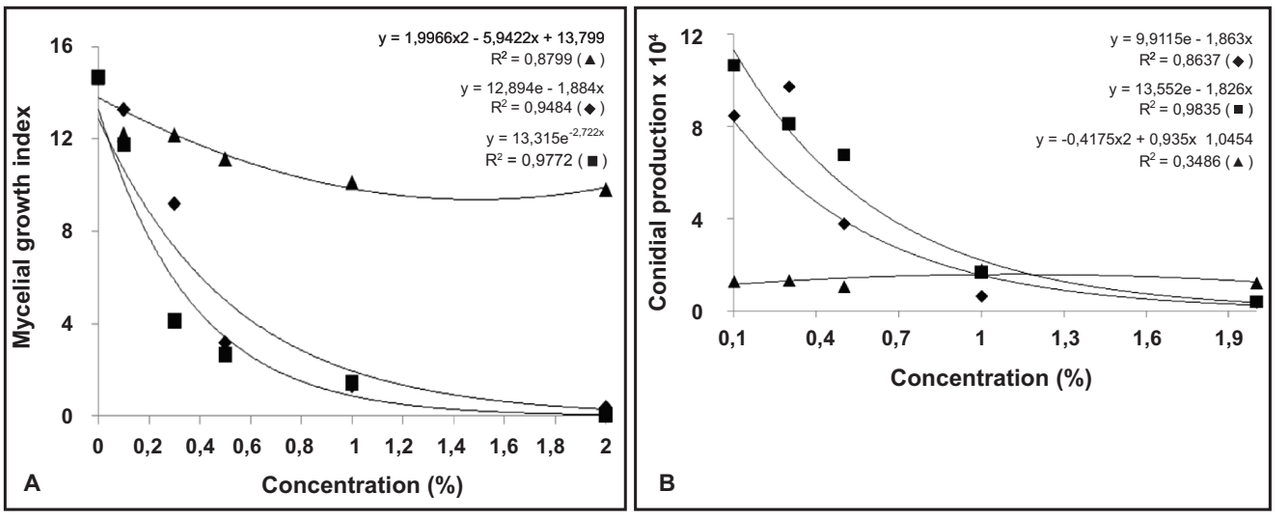

FIGURE 1 - Effect of essential oils on *Colletotrichum truncatum* mycelial growth and sporulation. **A.** Mycelial growth index of *Colletotrichum truncatum* in Petri dishes containing potato dextrose agar (PDA) supplemented with essential oils from *Aloysia gratissima* (■), *Hyptis marrubioides* (◆) and *Cordia verbenacea* (▲) at different concentrations. The mycelial growth index (MGI; mm/day) was calculated from the expression MGI = Σ(D –Da) / N, in which D is the actual mean colony diameter , Da is the previous mean diameter, and N is the number of days of growth; **B.** Effect of essential oils from (■) *Aloysia gratissima,* (◆) *Hyptis marrubioides* and (▲) *Cordia verbenacea* on the production of conidia by *C. truncatum*. The production of conidia was assessed by counting the number of spores in each Petri dish with a hemocytometer.

while aqueous extracts contain mostly glucans, pectins and tannins (Godard et al., 2009). Chromatographic analyses of the essential oils from *H. marrubioides*, *A. gratissima* and *C. verbenacea* employed in the present study revealed the presence of six main chemical classes, namely, alcohols, aldehydes, esters, ethers, hydrocarbons and ketones. It is likely that the antifungal activities of the oils are associated with synergism between the components, as has been suggested by other authors (Romagnoli et al., 2005; Sharma & Tripathi, 2006). The essential oil compounds are characteristic of each species. It is independent of the amount of material used for extraction. The composition can vary according to the season, time of day, growing conditions and genetic make-up of the plant (Perri et al., 1999; Tavares et al., 2005; Carvalho-Filho et al., 2006).

The present study has demonstrated that essential oils from *H. marrubioides*, *A. gratissima* and *C. verbenacea* were able to inhibit the sporulation of *C. truncatum* and reduce conidial germination and mycelial growth. Conidia production was inversely proportional to the concentration of essential oil applied. This result is interesting from an epidemiological standpoint, since the potential for plant infection by a pathogen is reduced when fungal reproduction is inhibited. Similar effects have been observed in relation to essential oils from a number of other plant species (Marques et al., 2004; Tolouee et al., 2010; Tian et al., 2011), including *Mentha spicata*, *Ricinus communis* and *Piper nigrum* (Ribeiro & Bedendo, 1999). On the other hand, an extract of *Allium sativum* did not affect significantly the production of conidia by *C. gloeosporioides* (Ribeiro & Bedendo 1999). According to Tzortzakis & Economakis (2007), inhibition of sporulation may result from a reduction in mycelial growth and/or from disruption by essential oils of the signal transduction pathways operating during the transition from vegetative to reproductive phases. Despite the effects on the sporulation of *C. truncatum* recorded in the present study, it is noteworthy that conidial viability was preserved during treatments with the essential oils. Marques et al. (2004) have previously reported that the viabilities of *Metarhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces farinosus* spores were not affected by treatment with neem (*Azadirachta indica*) oil.

The seed germination test showed that treatment of soybean seeds with essential oils did not have a negative effect on germination capacity. Indeed, some of the treatments increased germination with respect to non-inoculated non-treated seeds. Although similar results have been reported previously (Khan and Kumar, 1993; Souza et al., 2002; Brand et al., 2007; Koch et al., 2010; Steffen et al., 2010; Wulff et al., 2011), essential oils do not always exert a positive effect on seed quality. For example, the oils from *Cinnamomum zeylanicum* (Alves et al., 2004; Van der Wolf et al., 2008), *Eucalyptus citriodora* (Batish et al., 2004), and *Agastache rugosa* (Kim, 2008) inhibited, to varying extents, seed germination and seedling development.

The higher values of plant emergency observed in the greenhouse tests in relation to the values of the standard germination test run in laboratory, was also reported by França Neto & Henning (1984) for another pathogen,

Phomosis phaseolorum, in soybean seeds. It was noted that the contact between seed/seedlings and the pathogen is longer with the roll paper method than with the emergency tests done in the greenhouse, where the contaminated teguments are released as soon as seedlings emerge. This is why a higher percentage of seed-deteriorating pathogens and abnormal development is observed with the roll paper method.

In the present study, non-inoculated non-treated seeds presented low ESI values in comparison with their inoculated and treated counterparts. The probable reason for this finding is that the non-inoculated seeds were not submitted to osmotic pre-conditioning, which was part of the seed inoculation process, although the germination potential of soybean seeds is reportedly improved by such pre-conditioning (Giúdice et al., 1999).

The results presented herein demonstrate that oils from *H. marrubioides*, *A. gratissima* and *C. verbenacea* reduce the germination of conidia, the growth of mycelial and the reproduction of *C. truncatum,* although they do not affect conidial viability. The higher the concentration of the oils, the more effective the seed treatment was. At a concentration of 1%, the control of anthracnose by the essential oils was comparable to, or more effective than, that provided by the commercial fungicide carbendazim. Furthermore, the germination of soybean seeds was not affected negatively by any of the essential oils tested. It is concluded that the essential oils from these traditional Brazilian medicinal plants have potential as alternatives to synthetic pesticides for the control of anthracnose. It would be worthwhile to investigate the effect of the essential oils on other soybean pathogens.

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