Full Length Research Paper

Extraction of essential oil from inflorescences of *Dysphania ambrosioides* and its activity against *Botrytis cinerea*

Juliana Pace Salimena[1], Fernando Pereira Monteiro[1,2*], Paulo Estevão de Souza[1,2] and Jorge Teodoro de Souza[2]

[1]Department of Agriculture, Lavras Federal University, 37200-000 Lavras, MG, Brazil.
[2]Department of Phytopathology, Lavras Federal University. 37200-000 Lavras, MG, Brazil.

Essential oils are natural complex substances biosynthesized by plants, and many of them have antimicrobial properties. *Dysphania ambrosioides* is a medicinal plant traditionally used as an anthelmintic medicine. In this study, the antifungal activity of *D. ambrosioides* essential oil was tested against *Botrytis cinerea*, which is responsible for large economic losses in the post-harvest of roses. Inflorescences of *D. ambrosioides* yielded 1.3 mg.g^{-1} of essential oil in fresh material, corresponding to a content of 0.13%. Gas chromatography coupled with mass spectrometry (GC-MS) revealed 11 compounds in the essential oil with ascaridol and O-cymene corresponding to 80% of the total. The essential oil reduced *B. cinerea* mycelial growth by approximately 60% and spores germination by 51% at the concentration of 1000 ppm. However, no apparent morphological changes were observed in scanning electron microscopy analyzes. The essential oil was not able to reduce mycelial growth on rose petals and caused a color change in the petals 24 h after the treatment. Although the essential oil has little potential to control *B. cinerea* on roses due to the color change it causes, its activity on mycelial growth and spores germination could be exploited in other pathosystems.

Key words: *Rosa hybrida,* grey mold, *chenopodium ambrosioides*.

INTRODUCTION

Essential oils are natural plant products also known as volatile oils. They are fragrant substances with an oily consistency composed of a complex mixture of volatile molecules that may have multiple antimicrobial properties (Bassolé and Juliani, 2012). Some essential oils have activity not only by direct contact but also through the vapor phase, suggesting their use as fumigants (Umpiérrez et al., 2012). The use of fumigants avoids the direct contact with plants protecting them from possible harmful effects and ensures more security to the consumers (Shao et al., 2013; Umpiérrez et al., 2012). *Dysphania ambrosioides* (Chenopodiaceae sin.

Chenopodium ambrosioides) is a medicinal plant mainly used in folk medicine against intestinal parasites since immemorial times. The plant is native to Central and South America and has an annual or perennial cycle depending on the natural variety. This herb has a marked odor and is popularly known as worm seed, mastruz, goosefoot, paico or epazote (Lorenzi and Matos, 2002; Ortega-Ramirez et al., 2014). *D. ambrosioides* essential oil has toxic effects against fungi (Cabral et al., 2013), bacteria (Liu et al., 2013) and nematodes (Salifou et al., 2013).

The major compounds found in the essential oil of *D. ambrosioides* are p-cymene, carvacrol, isoascaridole and ascaridoles (Monzote et al., 2014; Ávila-Blanco et al., 2014). The ascaridoles are mainly responsible for its activity against fungi, including plant pathogens (Jardim et al., 2008; Zabka et al., 2009; Nisar et al., 2013). The development of natural fungicides with ascaridoles is encouraging due to its broad range of activity against several plant pathogens (Jardim et al., 2008). Other compounds in the oil may play a significant role in disease control because the complete essential oil has better activity in comparison with its pure major compounds (Monzote et al., 2014).

The essential oil of *D. ambrosioides* may be used as a natural fungicide in postharvest protection of agricultural commodities, such as cut flowers (Jardim et al., 2008). The distances between the cut flower production areas and consumer market may be long. For example, roses (*Rosa hybrida*) from South American countries are transported to the USA or Europe, while roses from Kenya and Ethiopia are transported via Western Europe to Eastern Europe and Asian countries (Harkema et al., 2013). Even at low transportation temperatures the roses may be damaged by fungi (Tuset, 1987).

The rose trade has great economic importance to Brazil. However, its competitiveness in the world market is greatly affected by the lack of research and improvement in areas such as resistance to pathogens (Barbieri and Stumpf, 2005). Roses are specially harmed by *Botrytis cinerea* (synonym: *Botryotinia fuckeliana*) that may attack more than 200 host species worldwide. Many fungicides have failed to control this pathogen due to its genetic plasticity (Williamson et al., 2007).

Excessive spraying of conventional chemicals used to control pests may harm the environment. Opposite to this view, there is a search for environmentally safe alternatives (Verma et al., 2009). Farmers have been using a variety of strategies against plant pathogens (Dominiak and Ekman, 2013). In addition, plant-derived compounds such as essential oils could also be applied.

The aims of this study were to determine the chemical composition and yield of the essential oil extracted from inflorescences of *D. ambrosioides,* and test its activity against *B. cinerea* pathogenic to roses. No previous reports were found on the use of *D. ambrosioides* essential oil in the control of rose diseases.

MATERIALS AND METHODS

D. ambrosioides cultivation

Dysphania ambrosioides was grown in tissue culture with standard procedures (Gangopadhyay et al., 2002). Five seedlings of approximately 5 mm long were inserted in a glass bottle containing MS basal medium (Murashige and Skoog, 1962) without hormone addition. The bottles remained for 40 days in a growth chamber with a photoperiod of 16 h of light and 8 h of darkness and temperature between 25 and 28°C. After this incubation period, the seedlings were transferred to a greenhouse and were acclimated for two weeks. After the acclimation, plants were transferred to boxes measuring 16 m long x 1.4 m wide x 1.1 m high filled with coarse sand and irrigated with a nutrient solution adjusted to pH 5.7±0.1. The composition of the nutrient solution used to fertilize *D. ambrosioides* was calcium nitrate (760 mg/L), ammonium sulfate (255 mg/L), monoammonium phosphate (110 mg/L), potassium chloride (400 mg/L), magnesium sulphate (375 mg/L), FeEDDHMA (44 mg/L), manganese sulphate (6.7 mg/L), boric acid (3.28 mg/L), zinc sulphate (2.96 mg/L), copper sulphate (0.36 mg/L) and sodium molybdate (0.05 mg/L). Plants were cultivated for 60 days in the sand box and harvested at flowering in 20 May, 2014, at 9 am in a mild day with no rainfall. The voucher specimen is deposited in the Biology Department herbarium of Lavras Federal University under accession number 10137.

Essential oil extraction and chemical composition

Essential oil extraction was performed by steam distillation (Marconi MA480). One hundred grams of fresh inflorescence was used for extraction. The hidrolact was collected after 90 min, and the essential oil was purified by liquid-liquid partition with 25 ml dichloromethane for three times. Organic fractions were combined and dried with anhydrous magnesium sulfate. The salt was removed by simple filtration and solvent was evaporated at room temperature in a gas exhaust chapel until constant weight.

After extraction of the essential oil each flask was transferred to amber glass bottles with stoppers and screw caps and weighed with an analytical balance (Marte Científica, Model AL500). Essential oil content was calculated with the following formula: Content (%) = (essential oil mass (g) x 100)/ fresh plant material weight (g). The analysis of chemical composition was done in triplicate. The quantitative analyzes were performed by chromatography coupled with a flame ionization detector hydrogen (GC-FID) and Agilent® 7890A system equipped with a fused silica capillary column gas-phase HP-5 (30 m long × 0.25 mm inside diameter x 0.25 mm thick film).

Helium was used as carrier at a flow of 1.0 ml/min. Injection and detection temperatures were maintained at 220°C and 240°C, respectively. Initial oven temperature was 40°C followed by a temperature ramp of 3°C/min up to 200°C and 10°C/min up to 250°C. The essential oil was diluted with 1% ethyl acetate and 1µL injected into the chromatograph using the split mode at 1:50. Quantitative analysis was obtained by integrating the total ion chromatogram and the content of the eluted constituents expressed as percentage of peak relative area.

Qualitative analyzes were done by gas chromatography coupled to mass spectrometry (GC-MS) with an Agilent® 5975C. This is operated in scan mode by electronic impact ionization at 70 eV at the speed of 1.0 scan/s and mass acquisition interval of 40 to 400 m/z. The chromatographic conditions were identical to the ones used in the quantitative analyzes. The components were identified by comparing their calculated Kovats retention indices (IK_c) with retention indices (IK) reported in the literature (Adams, 2007; Kováts, 1965) and by mass spectral comparisons. IK_c were calculated by applying the equation of Van Den Dool and Kratz

(1963) on the basis of standard n-alkanes co-injection C_8-C_{20} (Sigma Chemical Co.).

Effects of the essential oil on *B. cinerea*

B. cinerea was obtained from the CML mycological collection (Lavras Federal University, accession number 2317). Spores were produced on PDA medium at 25°C for eight days and suspensions were adjusted to 2×10^5 spores/ml. Spore suspensions with this concentration were used in all experiments described below.

Petri dishes divided in two sections were used to assess mycelial growth after treatment with the essential oil. On the center of one section containing 10 ml of PDA, an aliquot of 2 μL of the conidial suspension was added. In the other section, a cotton plug of approximately 0.16 g moistened with 1ml of a mixture of essential oil at 250, 500, 750 and 1000 ppm dissolved in 3% water-polysorbate (Tween 80) was placed. These cotton plugs were used to allow volatile dissipation for a long period of time. The controls were sterile distilled water-polysorbate at 3% in one section and the fungal spores in the other. The mycelial radial diameter was measured every 24h up to 96h. Mycelial growth rate was calculated according to Oliveira (1991): MGR=∑(D-Dp)/N where MGR=mycelial growth rate (cm.day^{-1}), D=current average diameter (cm), Dp=previous day average diameter (cm), and N=number of days after spore deposition. All experiments were installed in a completely randomized design with three replicates.

The effect on spore germination was assessed by using Petri plates divided in two sections. In one section containing 10 ml of 2% water-agar, 2 ml of the conidial suspension described above was added onto the medium surface. In the other section, a cotton plug moistened with 1 ml of a solution composed of 1% essential oil and 3% water-polysorbate was added. Control plugs were treated with 1 ml of water-polysorbate. Spores germination was evaluated every 24 h up to 96 h. Each treatment was assessed by adding 100 μL onto microscope slides and four randomized fields were assessed. Counting was repeated five times per treatment. The first 100 randomly selected conidia were counted and discriminated in germinated or non-germinated.

Scanning electron microscopy analyses were done on cultures grown for 3 days at 25°C in the presence of 1% essential oil either on cotton plugs as described above or with 100 μL applied directly onto the surface of the medium. Observations on possible alterations in the mycelium and conidia as affected by the essential oil were performed. Mycelial discs measuring 9 mm were collected from the plates, inserted in tubes with 1.5 ml of Karnovsky's fixative and stored at 4°C. After 24 h, each treatment was washed three times for 10 min in 0.05M cacodylate buffer solution, transferred to a 1% osmium tetroxide solution for 1h and washed with distilled water three times followed by dehydration in an acetone series (25, 50, 70, 90 and 100%). After dehydration, samples were submitted to the critical point dryer (Balzers CPD 030) to replace acetone by CO_2.

Specimens were placed on stubs covered with aluminum paper and carbon double-sided tape. Gold coating was done using a sputter coater (Balzers SCD 0.50) for observation with a scanning electron microscope LEO EVO 40 (Carl Zeiss AG). The images were recorded at 20 kV accelerating voltage and 10 mm of working distance.

Effect on mycelial growth on rose petals

A 5 mm diameter mycelial disk of *B. cinerea* was placed in the center of a detached rose petal. Petals were placed individually into Petri dishes in three replicates. Cotton plugs humidified with 1 ml of 1% *D. ambrosioides* essencial oil and controls with 1 ml of sterile distilled 3% water-polysorbate. Mycelial radial diameter was measured every 24 h up to 144 h.

Statistical analyses

All statistical analyses were done with the R software (R Core Team, 2014). Comparisons among means were performed using Tukey's test at 5% probability.

RESULTS AND DISCUSSION

Essential oil content and chemical composition

The yield and content of essential oil from *D. ambrosioides* inflorescences was 1.3±0.56 mg.g^{-1} and 0.13% in fresh material, respectively. In this study, the essential oil was extracted exclusively from inflorescences of *D. ambrosioides*. Other authors found essential oil contents varying from 0.3 to 1.8% when the whole aerial part was employed (Navaei and Mirza, 2004; Rondelli et al., 2012). These differences in oil content may be explained by water stress, growing season and stage of development at harvest, which may alter the chemical composition and yield (Morais, 2009).

Inflorescences appear to yield lower amounts of essential oil when compared to seeds and fruits that contain most of essential oil of the plant (Dembitsky et al., 2008). In fact, observations done in another study indicate that the yield of essential oil is approximately 9 times higher in fruits and seeds than in inflorescences (Unpublished data). The 11 identified chemical constituents represented 98.2% of the essential oil analyzed (Table 1).

The identity of the essential oil components was similar to those found in the literature, with exception of allyl hexanoate and cyclohex-2-enone,4-hydroxy-4-methyl which were found for the first time in the essential oil extracted from *D. ambrosioides*. The relative amounts of the components in the essential oil differed from the values found by other authors. The major components in inflorescences were ascaridole and O-cymene, corresponding to approximately 80% of the total (Table 1), while the major compounds in leaves were α-terpinene and O-cymene (Tapondjou, 2002; Singh et al., 2008). Comparatively, the essential oil extracted from inflorescences has more ascaridole than its precursor α-terpinene and may be more active against plant pathogens.

Volatile effect on mycelial growth and spore germination

There was a significant inhibition in mycelial growth of *B. cinerea* at 1000 ppm (Figure 1). Mycelial growth was inhibited by 59.8% and mycelial growth rate by 52.3% when compared to the control at the highest concentration of essential oil (1000 ppm). Higher

Table 1. Constituents of the essential oil extracted from inflorescences of *D. ambrosioides* identified by using gas chromatography.

Compound	Kovats index (Calculated)[1]	Kovats index (Literature)[2]	Content (%)[3]
α-terpinene	1013.58	1012	4.63
o-cymene	1021.62	1021	22.86
Sylvestrene	1025.13	1027	0.30
Allyl hexanoate	1072.64	1073	0.19
Cyclohex-2-enone,4-hydroxy-4-methyl	1120.58	1122	0.99
Ascaridole	1235.62	1237	57.28
<cis-> piperitone epoxide	1254.04	1254	7.36
Carvenone oxide	1264.96	1261	1.01
Thymol	1274.26	1274	0.17
Carvacrol	1292.74	1293	0.18
Iso-ascaridole	1301.20	1301	3.34
Total	-	-	98.21

[1]Kovats indices calculated by applying the equation of Van Den Dool and Kratz (1963) on the basis of standard n-alkanes co-injection C_8-C_{20} (Sigma Chemical Co.);[2] Kovats indices from Pubchem, Pherobase and ChemSpider databases; [3]Percentage of the compound when using a HP-5 column.
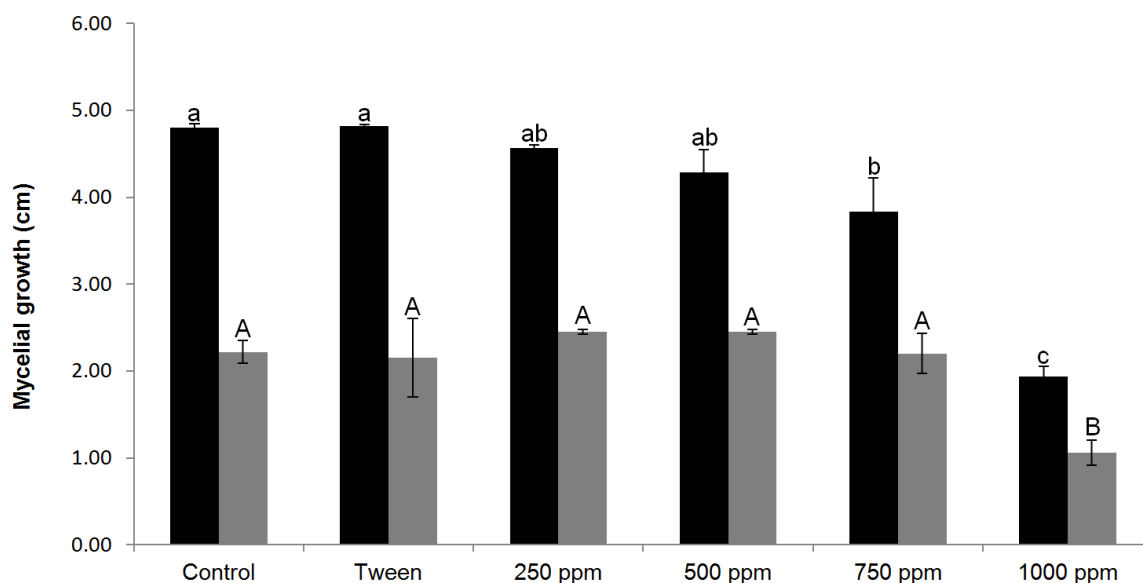

Figure 1. Effect of essential oil from inflorescence of *D. ambrosioides* on mycelial growth of *B. cinerea*. Black bars represent mycelial growth up to 72 h and grey bars represent mycelial growth rate (cm/day). Error bars represent standard error. Regression equation for mycelial growth considering the intervals between 0 and 1000ppm was estimated as following y=-0.002547x + 5.146667 (R^2=78.21).

inhibition was found by Tripathi et al. (2008) who observed 100% of mycelial growth inhibition at 500 ppm for *B. cinerea*. One possible reason for the differences may be the sensitivity of the isolates used in the different studies. Ascaridole, carvacrol and thymol are the only components with activity against *B. cinerea* mentioned in the literature (Jardim et al., 2008; Antunes and Cavaco, 2010). Although the purified components of the essential oil were not tested, they were found in this study. Spores germination was inhibited by essential oil volatiles up to 96 h after the treatment (Figure 2). Germination of spores was reduced by 58.3, 48.1 and 48.3% at 48, 72 and 96 h, respectively, after the application of the essential oil. The ethanolic extract of this plant was reported to inhibit spore germination by 18% in the closely related species *B. elliptica* (Hsieh et al., 2005). There were no morphological differences between spores or mycelium treated and untreated with essential oil (Figure 3). No other studies were performed with scanning electron microscopy to evaluate the effects of essential oil from
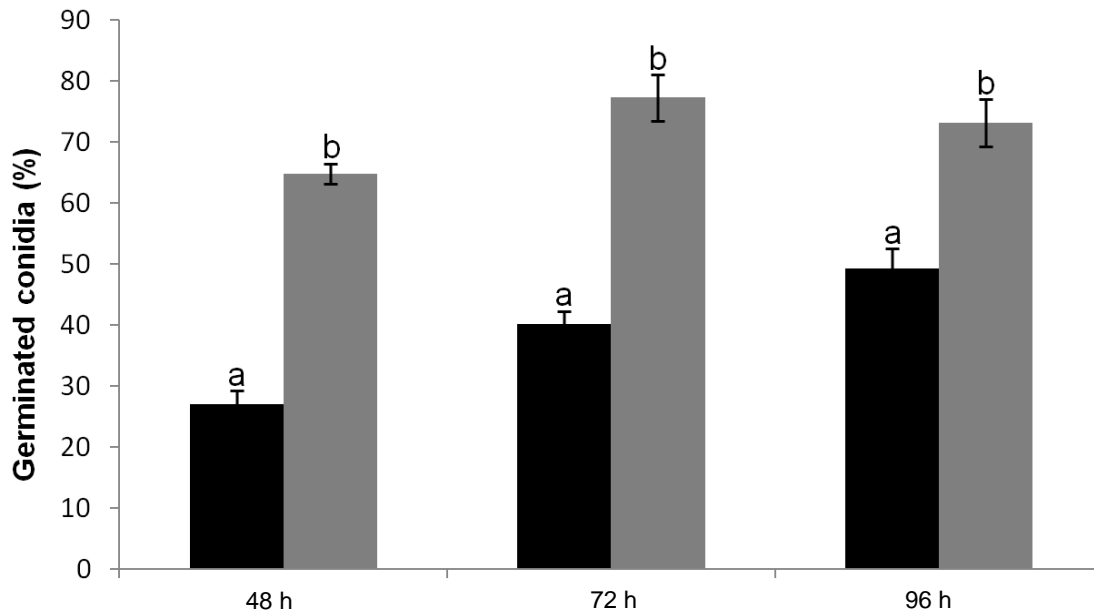
Figure 2. Effect of *D. ambrosioides* essential oil on *B. cinerea* spore germination. Black bars represent treatments and grey bars represent control. Error bars represents the standard error.
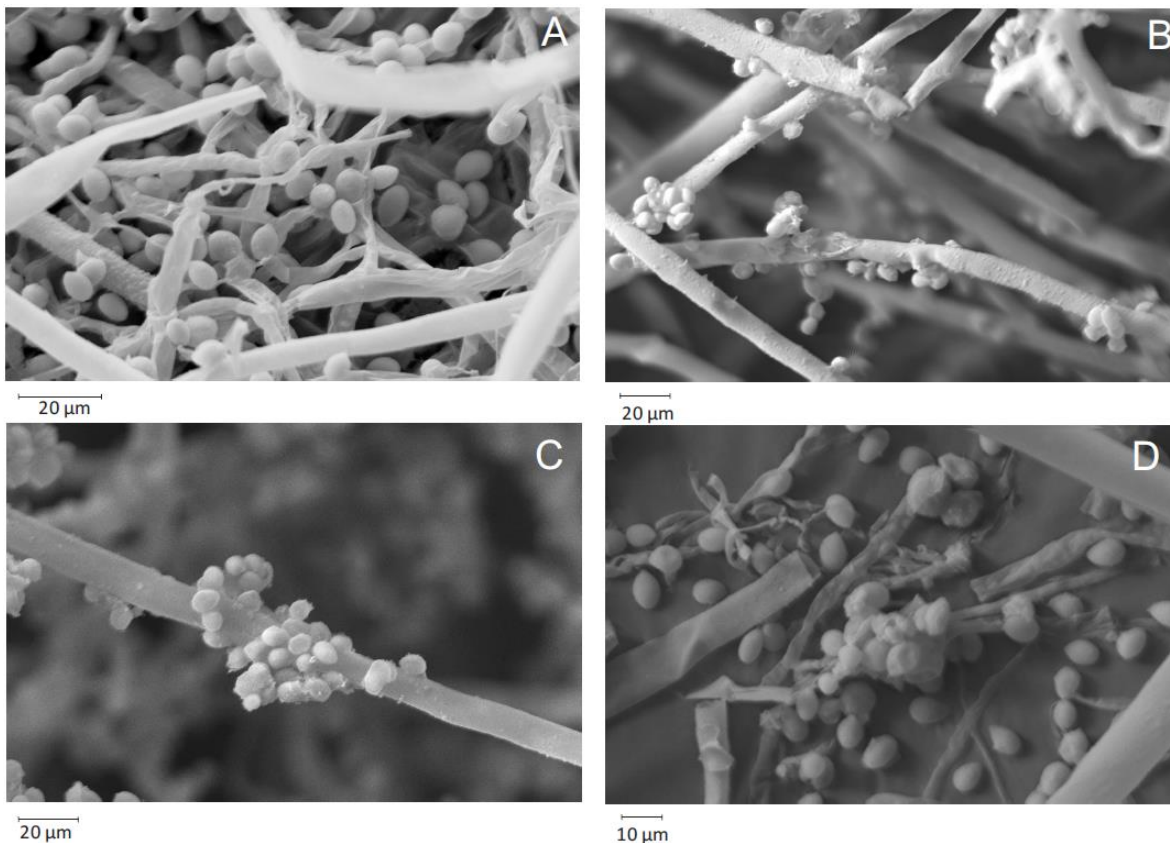

Figure 3. Scanning electron microscopy of the effect of D. *ambrosioides* essential oil on *B. cinerea*. **A** – *B. cinerea* grown in plates containing a cotton plug moistened with 1.6% water-polycarbonate (control); **B** - *B. cinerea* treated with 1% essential oil; **C** - *B. cinerea* with 1.6% water-polycarbonate applied directly on mycelial discs (control); **D** - 1% essential oil applied directly on mycelial discs.
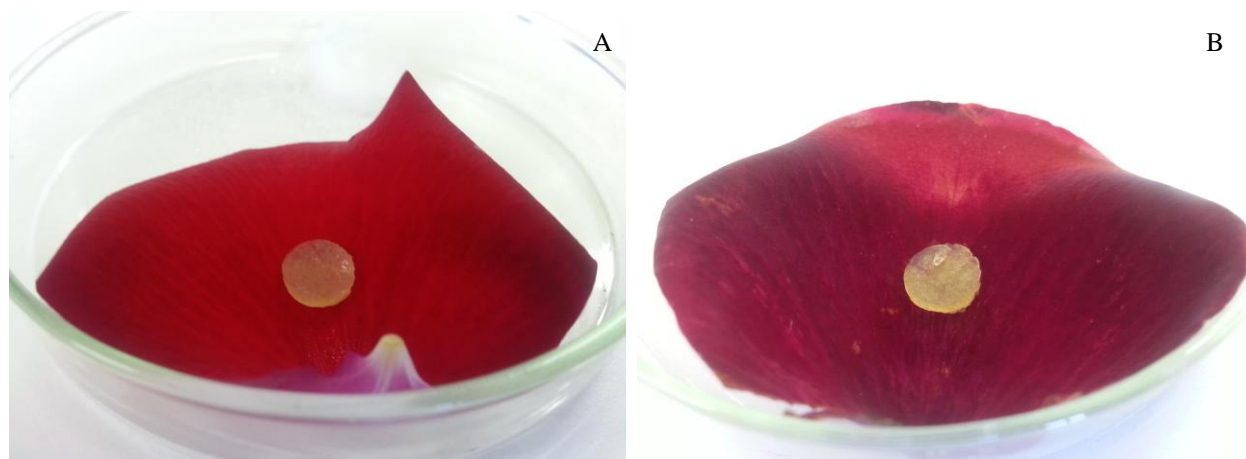
Figure 4. Effect of *D. ambrosioides* essential oil on detached rose petals. A - Control without treatment with essential oil; B - Rose petal treated with essential oil.

D. ambrosioides on mycelia and spores of *B. cinerea*.

Essential oil effect on *B. cinerea* mycelial growth on rose petals

There were no significant differences in mycelial growth among the treatments. The mycelial growth rate was 1.1cm.day^{-1}. The treatment with essential oil caused a color change in rose petals that could be observed after 24 h (Figure 4). The essential oil may have accelerated the senescence of the petals. This metabolic acceleration unfortunately limits the potential use of the essential oil from *D. ambrosioides* to protect roses against *B. cinerea*.

Conclusions

The essential oil of *D. ambrosioides* was successfully extracted from inflorescences, and its major compound was ascaridole. The essential oil reduced *B. cinerea* mycelial growth by 60% and spore germination by 51%. The essential oil did not control the development of *B. cinerea* on rose petals and caused a color change that is not acceptable in the commercial sector, but its use may be investigated for other pathosystems.

ACKNOWLEDGMENT

Conflict of interest

The authors have none to declare.

REFERENCES

Adams RP (2007). Identification of essential oils components by gas chromatography/ mass spectroscopy. Allured Publishing Corporation. pp. 1-469.

Antunes MDC, Cavaco M (2010). The use of essential oils for postharvest decay control. Flav. Frag. J. 25(5):351-366.

Ávila-Blanco ME, Rodríguez MG, Duque JLM, Muñoz-Ortega M, Ventura-Jaárez J (2014). Amoebicidal Activity of Essential Oil of *Dysphania ambrosioides* (L.) Mosyakin & Clemants in an Amoebic Liver Abscess Hamster Model. Evid. Based Compl. Altern. Med. 1:1-7.

Barbieri RL, Stumpf ERT (2005). Origin, evolution and history of cultivated roses. Rev. Bras. Agroc. 11(3):267-271.

Bassolé IHN, Juliani HR (2012). Essential oils in combination and their antimicrobial properties. Molecules 17(4):3989-4006.

Cabral LC, Pinto VF, Patriarca A (2013). Application of plant derived compounds to control fungal spoilage and mycotoxin production in foods. Int. J. Food Microbiol. 166(1):1-14.

Dembitsky V, Shkrob I, Lumir LO (2008). Ascaridole and related peroxides from the genus *Chenopodium*. Biomed. Pap Med. Fac. Univ. Palacky Olomouc Czech Repub. 152(2):209-15.

Dominiak BC, Ekman JH (2013). The rise and demise of control options for fruit fly in Australia. Crop Prot. 51:57-67.

Gangopadhyay G, Das S, Mukherjee KK (2002). Speciation in *Chenopodium* in West Bengal, India. Genet. Resour. Crop Evol. 49:503-510.

Harkema H, Mensink MG, Somhorst DP, Pedreschi RP, Westra EH (2013). Reduction of Botrytis cinerea incidence in cut roses (Rosa hybrida L.) during long term transport in dry conditions. Postharvest Biol. Technol. 76:135-138.

Hsieh TF, Huang JH, Hsieh LJ, Hu MF, Ko WH (2005). Antifungal effect of plant extracts on phytopathogenic fungi. Plant Pathol. Bull. 14(1):59-66.

Jardim CM, Jham GN, Dhingra OD, Freire MM (2008). Composition and antifungal activity of the essential oil of the Brazilian *Chenopodium*

ambrosioides L. J. Chem. Ecol. 34(9):1213-1218.

Kováts E (1965). Gas chromatographic characterization of organic substances in the retention index system. Adv. Chrom. 1:229-247.

Lorenzi H, Matos FJA (2002). Plantas medicinais no Brasil: nativas e exóticas. Editora Instituto Plantarum. pp. 1-512.

Liu W, Liu Y, Zhang X, Li N, Cheng H (2013). *In vitro* bactericidal activity of *Jinghua weikang* capsule and its individual herb *Chenopodium Ambrosioides* L. against antibiotic-resistant *Helicobacter Pylori.* Chin. J. Integr. Med. 19(1):54-57.

Monzote L, Pastor J, Scull R, Gille L (2014). Antileishmanial activity of essential oil from *Chenopodium ambrosioides* and its main components against experimental cutaneous leishmaniasis in BALB/c mice. Phytomedicine 21(8-9):1048-52.

Morais LAS (2009). Influência dos fatores abióticos na composição química dos óleos essenciais. Hort. Bras. 27(2):4050-4063.

Murashige T, Skoog, F (1962). A revised Medium for Rapid Growth and BioAssays with Tobacco Tissue Cultures. Phys. Plant 15(3):473-497.

Navaei MN, Mirza M (2004). Chemical composition of the oil of *Chenopodium ambrosioides* L. from Iran. Iran. J. Pharm. Res. 3(2):88-88.

Nisar M, Shah H, Khan I, Fazal-ur-Rehman, Khan MS, Marwat SK, Niazi ZR, Ullah A (2013). Antimicrobial Potential and Phytochemical Investigation of Fixed Oil of Plant Chenopodium ambrosioides Linn. Asian J. Chem. 25(2):1069-1072.

Oliveira JA (1991). Fungicide treatment effect on seeds in control of tipping cucumber seedlings (*Cucumis sativus* L.) and pepper (*Capsicum annum* L.). (Masters dissertation, Lavras Federal Universisty).

Ortega-Ramirez LA, Rodriguez-Garcia I, Leyva JM, Cruz-Valenzuela MR, Silva-Espinoza BA, Gonzalez-Aguilar GA, Siddiqui W, Ayala-Zavala JF. (2014). Potential of medicinal plants as antimicrobial and antioxidant agents in food industry: a hypothesis. J. Food Sci. 79(2):129-37.

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rondelli VM, Costa AV, Queiroz VT, Pinheiro PF, Pratissoli D (2012). Chemical composition and evaluation of the potential insecticide of *Chenopodium ambrosioides* essential oil in control of *Frankliniella schultzei*. Enc. Biosci. 8(15):2450-2458.

Salifou S, Daga DF, Attindehou S, Deguenon R, Biaou CF (2013). Antiparasitic effects of the water extract from *Chenopodium ambrosioides* L. (Chenopodiaceae) against some gastrointestinal nematodes in West African Long Legged goats. J. Paras. Vect. Biol. 5(2):13-16.

Shao X, Cheng S, Wang H, Yu D, Mungai C (2013). The possible mechanism of antifungal action of tea tree oil on *Botrytis cinerea*. J. Appl. Microb. 114(6):1642-1649.

Singh HP, Batish DR, Kohli RK, Mittal S, Yadav, S (2008). Chemical Composition of Essential Oil from Leaves of *Chenopodium ambrosioides* from Chandigarh, India. Chem. Nat. Comp. 44(3):378-379.

Tapondjou LA, Adler C, Bouda H, Fontem DA (2002). Efficacy of powder and essential oil from *Chenopodium ambrosioides* leaves as post-harvest grain protectants against six-stored product beetles. J. Stored Prod. Res. 38(4):395-402.

Tripathi P, Dubey NK, Shukla AK (2008). Use of some essential oils as post-harvest botanical fungicides in the management of grey mould of grapes caused by *Botrytis cinerea*. World J. Microbiol. Biotechnol. 24:39-46.

Tuset JJ (1987). Podedumbres de los frutos cítricos. Instituto Valenciano de Investigaciones Agrarias. pp. 1-206.

Umpiérrez ML, Lagreca ME, Cabrera R, Grille G, Rossini C (2012). Essential oils from Asteraceae as potential biocontrol tools for tomato pests and diseases. Phytochem. Rev. 11(4):339-350.

Van Den Dool H, Kratz DJ (1963). A generalization of the retention index system including liner temperature programmed gas-liquid partition chromatography. J. Chromatogr. 11:463-467.

Verma M, Sharma S, Prasad R (2009). Biological alternatives for termite control: A review. Int. Biodeterior. Biodegrad. 63(8):959-972.

Williamson B, Tudzynski B, Tudzynsk P, Van Kan JAL (2007). *Botrytis cinerea*: the cause of grey mould disease. Mol. Plant Pathol. 8(5):561-80.

Zabka M, Pavela R, Slezakova L (2009). Antifungal effect of *Pimenta dioica* essential oil against dangerous pat hogenic and toxigenic fungi. Ind. Crop Prod. 30(2):250-253.