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Essential oils from *Hyptis marrubioides*, *Aloysia gratissima* and *Cordia verbenacea* reduce the progress of Asian soybean rust

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ABSTRACT. The aim of this study was to evaluate the potential of essential oils derived from *Hyptis marrubioides*, *Aloysia gratissima* and *Cordia verbenacea* for controlling Asian soybean rust. The inhibitory activities of the essential oils (applied in a concentration range of 0.05 - 2%) on the germination of *Phakopsora pachyrhizi* urediniospores were investigated using *in vitro* assays. The curative and eradicating properties of the oils (applied in a concentration range of 0.05 - 0.5%) were studied under greenhouse conditions using the *P. pachyrhizi*-susceptible soybean cultivar MG/BR 46 (Conquista). Scanning electron microscopy was employed to investigate the effects of the essential oils on the morphology of the *P. pachyrhizi* urediniospores. The treatment with the essential oils at all concentrations tested led to the total inhibition of spore germination *in vitro*. The curative application of the essential oils reduced the disease severity, expressed as the area under the disease-progress curves, by 33 to 41%, whereas the commercial fungicide (pyraclostrobin + epoxyconazole) employed as a positive control reduced the severity by 61%. The treatment of infected plants with the essential oils resulted in morphological alterations in the fungal structures that were similar to those produced by the commercial fungicide, namely, a shrivelling of the urediniospores, appressoria, germ tubes and paraphyses.

Keywords: *Glycine max*, *Phakopsora pachyrhizi*, alternative disease control, scanning electron microscopy.

Óleos essenciais de *Hyptis marrubioides*, *Aloysia gratissima* e *Cordia verbenacea* reduz o progresso da ferrugem asiática da soja

RESUMO. O objetivo do trabalho foi avaliar o potencial dos óleos essenciais extraídos de *Hyptis marrubioides*, *Aloysia gratissima* e *Cordia verbenacea* no controle da ferrugem asiática da soja. A atividade inibitória dos óleos essenciais (aplicados no intervalo de concentração de 0,05 - 2%) sobre a germinação dos urediniósporos de *Phakopsora pachyrhizi* foram avaliados em teste *in vitro*. As propriedades curativas e erradicantes dos óleos (aplicados no intervalo de concentração de 0,05 - 0,5%) foram estudadas em casa de vegetação utilizando a cultivar MG/BR 46 (Conquista) suscetível a *P. pachyrhizi*. Microscopia eletrônica de varredura foi utilizado para investigar o efeito dos óleos essenciais sobre a morfologia dos urediniósporos do fungo. Os tratamentos com os óleos essenciais, em todas as concentrações testadas inibiram totalmente a germinação dos urediniósporos *in vitro*. A aplicação curativa dos óleos essenciais reduziu a severidade da doença, expressa em área abaixo da curva de progresso em 33 a 41%, enquanto que o fungicida comercial (piraclostrobina + epoxiconazole), empregado como controle positivo reduziu em 61%. Tratamento de plantas infectadas com óleos essenciais produziu alterações morfológicas nas estruturas fúngicas, semelhantes as produzidas pelo fungicida comercial, ou seja, murchamento dos urediniósporos, apressórios, tubos germinativos e paráfise.

Palavras-chave: *Glycine max*, *Phakopsora pachyrhizi,* controle alternativo de doenças, microscopia eletrônica de varredura.

Introduction

The yield and profitability of a soybean crop can be significantly affected by diseases caused by plant pathogens. The Asian soybean rust caused by the fungus *Phakopsora pachyrhizi Syd. & P. Syd* (YANG et al., 1990, 1991) is one of the most destructive diseases in soybean. The initial symptoms include the appearance of small chlorotic areas that evolve into necrotic areas on the surfaces of the leaves. In the absence of control, the disease intensity can increase to severe levels and lead to premature defoliation that, depending on the crop development, can cause abnormal pod production, with reduced seed fill and smaller seed size (YANG et al., 1991). According to studies conducted in various soybean-growing regions, the reductions in yield caused by Asian rust can vary between 10 and 100% (HARTMAN et al., 1991; YORINORI et al., 2005; MCLAREN, 2008). In Brazil, it has been estimated that the cumulative total economic losses, including reduced yield and revenue plus the cost of disease control, due to soybean rust have reached US $ 10.1 billion since the first widespread epidemics were observed in the country (CONSÓRCIO ANTIFERRUGEM, 2008).

Currently, no commercial soybean cultivars have adequate levels of resistance against soybean rust (NAVARINI et al., 2007; RIBEIRO et al., 2007), and, hence, the control of the disease is obtained using integrated measures. Once an infection is established, the use of a commercial mixture of triazole and strobilurin fungicides is unavoidable (PATIL; ANAHOSUR, 1998; MILES et al., 2003; GODOY; CANTERI, 2004). However, the continuous use of such specific fungicides may favour the selection of pathogen-resistant strains; therefore, it is necessary to alternate such products with other products that act via different mechanisms (KIMATI, 1987).

Plant-based products both provide alternatives to the synthetic fungicides and their application is generally less harmful to both people and the environment. The essential oils from a variety of plant species have been shown to be very effective in the control of plant diseases, such as those caused by *Phakopsora pachyrhizi*, *Colletotrichum gloeosporioides*, *Botrytis cinerea*, *Colletotrichum capsici*, *Fusarium oxysporum*, *Fusarium solani*, *Phytophthora capsici*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (MEDICE et al., 2007; SILVA et al., 2009; AL-REZA et al., 2010). Furthermore, the oils from traditional Brazilian medicinal plants, including *Hyptis marrubioides* Epling ex Hoehne (Lamiaceae), *Aloysia gratissima* (Gillies and Hook.) Tronc. (Verbenaceae) and *Cordia verbenacea* DC. (Boraginaceae), are known to be effective in the control of anthracnose in soybean seeds (SILVA et al., 2012a). To evaluate the efficacy of the essential oils from these plant species in disease control, this work aimed to investigate the curative effects of essential oils derived from these plants against Asian soybean rust.

Material and methods

Preparation of essential oils

Plants of *H. marrubioides*, *A. gratissima* and *C. verbenacea* were grown in the medicinal garden at the Universidade Federal de Lavras (UFLA) campus, Lavras, MG, Brazil. Voucher specimens were deposited in the herbarium at UFLA, with reference numbers 1022, 19810 and 7982, respectively. The aerial parts (leaves and flowers) of each plant were collected during the mornings in February 2008 and were immediately comminuted and transferred to a modified Clevenger apparatus. Hydrodistillation was conducted for 2h, and the essential oil collected was stored at -40°C in aluminium foil-wrapped glass vials until it was required for the assays.

Preparation of *P. pachyrhizi* urediniospores

Urediniospores were scraped from the leaves of naturally infected soybean plants growing in the fields of the university campus. The spores were passed through a 60 mesh sieve, collected onto aluminium foil and subsequently suspended in distilled water containing 1% Tween 20. The spore density was adjusted by an appropriate dilution after counting using a light microscope with the aid of a Neubauer chamber. Spore viability was evaluated prior to inoculation onto the growth medium.

Effects of essential oils on the germination of *P. pachyrhizi* spores *in vitro*

The essential oils were separately mixed with 1% Tween 20 in water to produce concentrated emulsions, and aliquots (1 mL) were combined with 9 mL of autoclaved water-agar medium, maintained just above the melting point of agar, and immediately poured into 9 cm diameter Petri dishes. The final concentrations of the oils in the media were 0.05, 0.1, 0.3, 0.5, 1.0, 1.5 and 2.0%. Following the solidification of the medium, an aliquot (50 μL) of spore suspension (containing 2 x 10^4 spores mL^{-1}) was placed onto the agar and spread evenly across the surface, and the plates were sealed. The inoculated dishes were incubated at 25°C for 4h in the dark, and the number of germinating spores was counted in samples of 200 spores located in each quadrant of the dish. A spore was considered to be germinated when the length of its germ tube was equal to or greater than the spore diameter.

The assays were conducted in triplicate, with each dish representing a replicate, according to a completely randomised design, with three different essential oils x seven different oil concentrations, plus two controls. In the negative (untreated) control, a 1 mL aliquot of water containing 1% Tween 20 replaced the essential oil emulsion; in the positive control, the test emulsion was replaced with a 1 mL aliquot of fungicide solution containing 2.22 mg of pyraclostrobin and 0.83 mg of epoxyconazole (Opera®, BASF).

Effects of essential oils on *P. pachyrhizi*-infected soybean plants *in vivo*

To assess the curative and eradication properties of the essential oils, *in vivo* assays were performed using soybean plants that had been artificially infected with Asian rust. Seeds of the *P. pachyrhizi*-

susceptible soybean cultivar MG/BR 46 (Conquista) were germinated in plastic pots, with each pot containing 3 kg of substrate (soil:sand:manure; 2:1:2 by weight). After 30 days, the seedlings were thinned, leaving two plantlets per pot. After 40 days, a cover fertilizer was applied following the recommendations of Novais et al. (1991). At the start of flowering (phenological stage R1), the plants were inoculated with a suspension of *P. pachyrhizi* urediniospores (containing 2×10^5 spores mL^{-1}) and subsequently enclosed in transparent plastic bags for 12 hours to provide a saturated humid environment. The inoculated plants were then maintained in a greenhouse with the temperature and humidity constantly monitored with the aid of an Impac (São Paulo, São Paulo State, Brazil) model TH 508 thermohygrograph. The conditions employed (average temperature in the range 17.5-30.2ºC; relative humidity of 74.6%) were considered favourable for the development of soybean rust (KOCHMAN, 1979).

The assay of the essential oils commenced on the 14th day after inoculation of the plants. Four pairs of trifoliolate leaves were selected near the centre of each plant, comprising two pairs that showed no signs of disease and two pairs that demonstrated a low level of disease severity (the minimum level according to the diagrammatic scale proposed by GODOY et al., 2006). These leaves were marked and subsequently re-evaluated for disease severity (according to the scale mentioned above) at 7 days intervals. A total of seven evaluations were performed. The area under the disease progress curve was calculated according to the method of Shaner and Finney (1977). The plants were sprayed until runoff with the essential oils at concentrations of 0.05, 0.1, 0.3 and 0.5% (as emulsions in water containing 1% Tween 20) every 10 days following the first evaluation using a manual atomiser. The control plants were treated in exactly the same manner but with either 1% Tween 20 in water (negative control) or a commercial fungicide mixture consisting of pyraclostrobin + epoxyconazole (Opera®), at a dosage of 66.5+25 g i a ha^{-1} (positive control) replacing the essential oil emulsion. The assays were conducted in quintuplicate, with each pot representing a replicate, according to a randomised block design with three different essential oils x four different oil concentrations, plus two controls. The complete experiment comprised 70 pots, with each pot containing two plants.

Scanning electron microscopy (SEM)

The effects of the essential oils on the morphology of the *P. pachyrhizi* urediniospores were evaluated by SEM. In the greenhouse, soybean plants that were severely infected (18% infected leaf area according to the diagrammatic scale proposed by GODOY et al., 2006) with *P. pachyrhizi* were treated with the essential oils at concentrations of 0.05, 0.1, 0.3 and 0.5% (as emulsions in water containing 1% Tween 20), as described above. The control plants were treated with either 1% Tween 20 in water (negative control) or the above commercial fungicide mixture (positive control). At seventeen hours after treatment, the inoculated trifoliolate leaves were removed from the plants and prepared for SEM analysis following the methodology described by Medice et al. (2007).

Statistical analysis

All of the statistical analyses were performed using R software (R Development Core Team). All of the assays were performed twice. The data were subjected to an analysis of variance (ANOVA): the normality and homogeneity of variance were evaluated through the inspection of the residual plots, and no deviations from the assumptions were observed. Tukey tests ($\alpha = 0.05$) were applied to determine the significance of the differences between the mean values.

Results and discussion

The incubation of the *P. pachyrhizi* urediniospores on a water-agar medium supplemented with 1 mL of water containing 1% Tween 20 (negative control) allowed 74% spore germination. In contrast, the addition of 1 mL of a water:Tween 20 emulsion of the essential oils from *H. marrubioides*, *A. gratissima* or *C. verbenacea* led to the total inhibition of spore germination, irrespective of the oil concentration. The commercial fungicide also inhibited germination by 100%. These results demonstrate that essential oils from the traditional Brazilian medicinal plants *H. marrubioides*, *A. gratissima* and *C. verbenacea* contain components that are toxic to the urediniospores of *P. pachyrhizi*. A 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*, such as alcohols, aldehydes, esters, ethers, hydrocarbons and ketones (SILVA et al., 2012a). It is likely that the antifungal activities of the oils are associated with a synergism between the components, as has been suggested by other authors (ROMAGNOLI et al., 2005; SHARMA; TRIPATHI, 2006).

ANOVA revealed that there were no double (Leaf : oil; Leaf : concentration; Oil : concentration) or triple

(Leaf : oil : concentration) interactions among the factors when studied *in vivo* and that each factor acted independently. Regarding the curative effects of the different essential oil concentrations, no significant differences were detected between any of the treatments applied: even at the lowest concentration (0.05%), the oils studied were able to control the disease. The results also demonstrated that the essential oils from the three species studied were equally effective in controlling the soybean rust (Table 1). These results were also observed in the control of powdery mildew in Eucalyptus (SILVA et al., 2014). A result that is significant because it suggests that these essential oils could be employed in the control of rust in the field. Moreover, it would appear that oil concentrations lower than the minimum levels assayed in the present study might also be effective.

Table 1. Mean values for the area under the disease progress curve *(AUDPC)* obtained using essential oils as curative and eradicative treatments in the control of Asian soybean rust.

Treatment	AUDPC values		
	Mean	Lower	Upper
Aloysia gratissima oil	106.5 [b]	96.1	116.8
Cordia verbenacea oil	100.0 [b]	89.6	110.4
Hyptis marrubioides oil	113.3 [b]	102.9	123.7
Commercial fungicide (positive control)	66.0 [a]	45.2	86.7
1% Tween 20 in water (negative control)	169.0 [c]	148.2	189.7

Within a column, the values denoted with different lower-case superscript letters are significantly different at a 5% probability (Tukey test).

Although considerable research has been focused on the effects of essential oils on plant pathogens, studies involving the use of such natural products in the control of soybean rust are scarce. Reports concerning the antifungal properties of the essential oils from *A. gratissima*, *C. verbenacea* and *H. marrubioides* are also limited. Medice et al. (2007) reported that the application of the essential oils from *Corymbia citriodora, Cymbopogon nardus*, *Azadirachta indica* and *Thymus vulgaris* to the soybean cultivars Conquista and Suprema reduced the density of rust pustules on infected leaves. In contrast, the application of citrus extracts to soybean plants was ineffective against *P. pachyrhizi* infection, though it was successful in reducing the severity of *Peronospora manshurica* (62%) and *Microsphaera diffusa* (35%) infections (KUHN et al., 2009). Silva et al. (2012b) have previously demonstrated that the preventative spraying with the essential oils from *H. marrubioides*, *A. gratissima* and *C. verbenacea* was effective in controlling *P. pachyrhizi* infections. In the present study, the oils were found to be efficient at very low concentrations (0.05%), in contrast to the findings of Silva et al. (2012b) who reported that their efficacy in the prevention of soybean rust improved with increasing concentrations.

Moreover, it was reported that the preventative application of the essential oils reduced the progress of rust disease and afforded 52 to 80% protection in comparison with the 92% effectiveness provided by the commercial fungicide employed as the positive control. The curative application of the essential oils imparted 33 to 41% protection, whereas the fungicide mixture was 61% effective. According to Hartman et al. (1991), the preventative application of fungicides represents the most appropriate strategy for controlling soybean rust, a hypothesis that is confirmed by the reported effects of the commercial fungicide used as the positive control in the studies mentioned above. Additionally, the essential oils in the present study were more effective when applied in a prophylactic manner rather than for curative purposes. However, it is difficult to detect soybean rust at an early stage because the initial symptoms can be confused with those of other diseases (LEVY, 2005). For this reason, it is essential to investigate products that might be used in the treatment of rust-infected crops to minimise the consequential economic losses.

Although the essential oils exerted a curative effect on the soybean plants, none was able to impede the development of *P. pachyrhizi* or to eradicate the disease completely. It is of significance to note that systemic fungicides (azoxystrobin, carbendazim, tebuconazole, difenoconazole and a epoxyconazole/pyraclostrobin mixture) are also incapable of completely eradicating soybean rust (GODOY; CANTERI, 2004). However, it was noted that the leaves that exhibited no signs of rust on the 14th day after inoculation and that received an essential oil treatment every 10 days showed less progression of the disease when compared with the leaves that had already presented signs of rust (Figure 1).

Figure 1. Comparison of the mean values of the areas under the disease progress curves (AUDPC) of the soybean leaves that presented rust symptoms with those that exhibited no symptoms at the first evaluation following the treatment with the essential oils. The bars labelled with the different lower-case letters indicate mean values that are significantly different one from another (Tukey test; 5% probability).

Indeed, a number of studies have shown that the curative efficacy of fungicides generally diminishes when the application is delayed (MILES et al., 2003; GODOY; CANTERI, 2004), and the results of Scherm et al. (2009) confirm that fungicides should be applied prior to the emergence of soybean rust, or at the latest when the first signs of infection become visible, otherwise the success of treatment could be reduced.

Generally, however, the application of fungicides during the vegetative growth stage is ineffective and uneconomical (MILES et al., 2003). In the present investigation, the essential oil treatments were applied during the reproductive R1 stage, which is considered a critical period during which soybean plants are particularly susceptible to *P. pachyrhizi* infection.

In the present study, a commercial fungicide containing pyraclostrobin and epoxyconazole (positive control) was more effective as a curative treatment against soybean rust than were any of the essential oils assayed. Pyraclostrobin is a strobilurin fungicide that acts systemically (translaminar) by disrupting the early events in fungal development, i.e., spore germination and the elongation of the germ tube (BARTLETT et al., 2002). Epoxyconazole is a triazole fungicide that affects later stages of fungal development, such as mycelial growth and the fungal cycle (O'LEARY; SUTTON, 1986; TSUDA et al., 2004). In contrast, plant-derived fungicides are generally comprised of multi-component combinations that exhibit a variety of mechanisms of action (SHARMA; TRIPATHI, 2006), which may include direct toxicity (MEDICE et al., 2007; SILVA et al., 2009; AL-REZA et al., 2010; CHAIJUCKAM; DAVIS, 2010), stimulation of the mechanisms of plant resistance (DEVAIAH et al., 2009; GODARD et al., 2009) and systemic action on the plant cells (SHIVASHANKAR et al., 2000; ITAKO et al., 2008). The lower efficacy of the essential oils in comparison with the commercial fungicide may be related to the high volatility and rapid degradability of the former when exposed to the atmosphere, intense light and/or high temperature (SIMÕES; SPITZER, 2000; RUDDICK, 2008).

The treatment of the infected plants with the essential oils induced morphological alterations in the fungal structures that were similar to those produced by the commercial fungicide. Even the lowest concentration of the essential oils gave rise to urediniospores, appressoria, germ tubes and paraphyses that were shrivelled in comparison with those observed in the infected plants that had been treated only with water containing 1% Tween 20 (Figure 2).

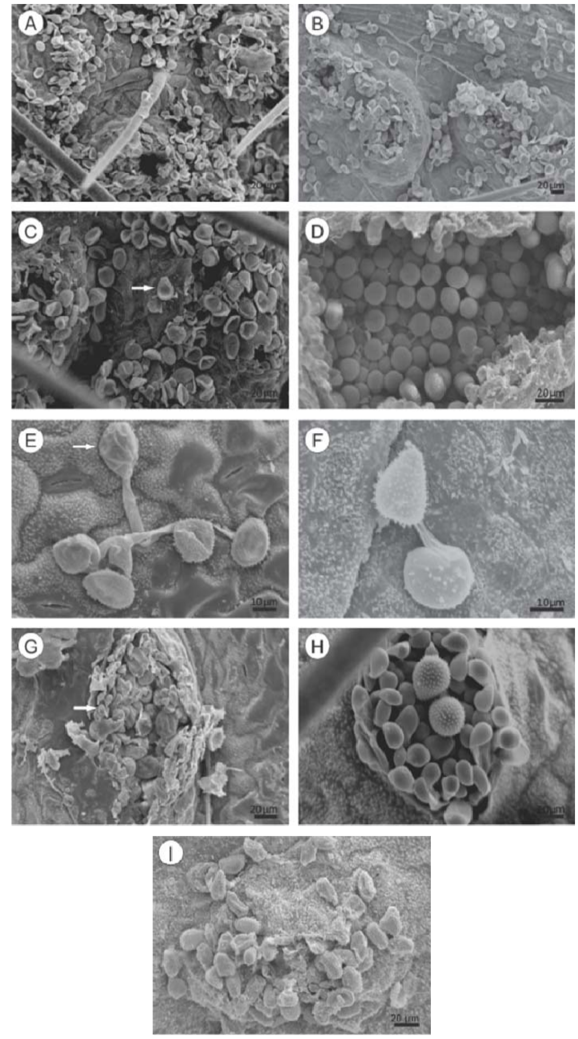

Figure 2. Scanning electron microscope images of fungal structures on the leaves of infected soybean plants that had been treated with 0.05% emulsions (in water containing 1% Tween 20) of the essential oils from *Aloysia gratissima* (A), *Cordia verbenacea* (B, E and G) and *Hyptis marrubioides* (C). The arrows indicate the shrivelled urediniospores (C), appressoria (E) and paraphyses (G). Similar alterations were observed in the fungal structures on the leaves of infected soybean plants that had been treated with higher concentrations of essential oils and with a commercial fungicide (I), but no morphological changes were detected in the negative controls that had been treated with water containing 1% Tween 20 but no additive (D, F and H).

The treatment of the artificially infected plants with these essential oils or with a commercial fungicide containing pyraclostrobin and epoxyconazole inhibited fungal germination and induced morphological changes in the fungal structures that were not detected in the untreated (negative) controls. The observed alterations were in agreement with previous studies (SOYLU et al., 2007; MEDICE et al., 2007) involving the effect of oils from different plant species and on fungi other than *P. pachyrhizi*. It has been suggested that such alterations result from cytoplasmic leakage and from the disruption of the enzyme-mediated synthesis of fungal cell walls caused by lipophilic oils (SHARMA; TRIPATHI, 2006; SOYLU et al., 2007).

Although the essential oils studied were unable to eradicate *P. pachyrhizi* infection completely, it was possible to observe a significant curative effect in terms of diminished disease progress in the treated plants grown under greenhouse conditions, alterations in the fungal development, as revealed by SEM, and reduced spore germination, as established by an *in vitro* assay.

The results presented herein establish the potential of the essential oils from *H. marrubioides, A. gratissima* and *C. verbenacea* in curative treatments against *P. pachyrhizi* infection. These natural products are good alternatives to the use of synthetic fungicides, but could also have applications in both organic and conventional farming. It would be of interest to establish different formulations of these oils to allow the retention of the residual activity and to investigate their efficacy against other soybean pathogens.

Conclusion

The essential oils from *H. marrubioides*, *A. gratissima* and *C. verbenacea* inhibit the germination of *P. pachyrhizi* urediniospores.

The essential oils from the three species studied were equally effective in controlling Asian soybean rust, though they were less efficient than the commercial fungicide.

The curative application of the essential oils reduces the progress of Asian soybean rust. The essential oils should be applied curatively when the first signs of infection become visible, otherwise the success of the treatment could be reduced.

The treatment of infected plants with the essential oils produces morphological alterations in the fungal structures that are similar to those produced by the commercial fungicide.

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