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Essential oils and whole milk in the control of soybean powdery mildew

Óleos essenciais e leite integral no controle do oídio da soja

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ABSTRACT

*This research aimed to evaluate the potential of essential oils (EOs) and cow's whole milk (CWM) in order to control soybean powdery mildew and to estimate the most effective concentrations of these natural products in reducing the disease severity on soybean plants. Three experiments were carried out: The first experiment evaluated and selected the most effective treatments to reduce the severity of soybean powdery mildew under greenhouse conditions; the second experiment evaluated the effect of CWM and EOs of citronella, lemongrass, eucalyptus, cinnamon and tea tree on the pathogen through the ultrastructure analysis of soybean leaflets infected by **Erysiphe diffusa** using the scanning electron microscope (SEM) and light microscope (LM) technology. In the third experiment, the most effective products were tested at several concentrations in order to define the most effective concentrations to reduce disease severity under greenhouse conditions. The treatments CWM (100mL L^{-1}) and EOs of citronella, lemongrass and eucalyptus (1.0mL L^{-1}), reduced the disease severity from 67 to 74%. Direct effects from all natural products tested on the structures of **E. diffusa** were demonstrated through the SEM and LM analysis. Concentrations at 1.5mL L^{-1} for EOs of citronella, lemongrass and eucalyptus and also at 180mL L^{-1} for the treatment CWM were the most effective against **E. diffusa** on soybean.*

Key words: ***Erysiphe diffusa***, ***Glycine max***, *Essential oil as biocontrol, scanning electron microscopy, effective concentrations*.

RESUMO

*O objetivo deste trabalho foi selecionar e avaliar o potencial de óleos essenciais (OEs) e leite de vaca integral (LVI) no controle do oídio da soja e determinar as concentrações mais efetivas destes produtos na redução da severidade dessa doença. Foram realizados três experimentos: um experimento em casa de vegetação para avaliação e seleção dos produtos mais efetivos na redução da severidade do oídio da soja; um experimento para a verificação do efeito do LVI e OEs de citronela, capim-limão, eucalipto, canela e árvore-de-chá sobre o patógeno, por meio da análise ultraestrutural de folíolos infectados por **Erysiphe diffusa** em microscópio eletrônico de varredura (MEV) e de luz (ML); e finalmente um experimento em casa de vegetação, utilizando os produtos mais ativos, em diferentes concentrações, com o propósito de determinar as concentrações efetivas na redução da severidade da doença. Os tratamentos LVI (100mL L^{-1}) e OEs de citronela, capim-limão e eucalipto a 1.0mL L^{-1}, apresentaram controle de 67 a 74% da doença. Por meio da análise ultraestrutural em MEV e ML, foi evidenciado um efeito direto sobre as estruturas de **E. diffusa**, por parte de todos os produtos naturais testados. Concentrações de 1.5mL L^{-1}, para os OEs de citronela, capim-limão e eucalipto e de 180mL L^{-1} para o LVI foram mais efetivas contra **E. difusa** em soja.*

Palavras-chave: ***Erysiphe diffusa***, ***Glycine max***, *controle biológico, microscopia eletrônica de varredura, concentrações efetivas.*

INTRODUCTION

Brazil is currently the second largest soybean [***Glycine max*** (L.) Merr.] producer after the United States. Among the diseases with economic importance to Brazil, powdery mildew [***Erysiphe diffusa*** (Cooke & Peck) U. Braum & Takam.] is highlighted, especially in the 'cerrado' area. Powdery mildew on soybeans became important in Brazil after an outbreak occurred in all cultivated areas, in the

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1996/97 season. Since then, the disease has become a constant threat to farmers, causing losses of up to 25% in crop yield (ALMEIDA et al., 2008).

According to SARTORATO & YORINORI (2001), the most efficient and economical method to powdery mildew control is the use of resistant cultivars. However, due to the large pathogenic variability of ***E*. *diffusa***, some cultivars may have their resistance overcome by the pathogen and resistant cultivars are still not under availability. Thus, chemical control is the most widely used method. Nevertheless, the indiscriminate use of these products leads to problems such as selection of resistant strains of the pathogens; furthermore, these methods contaminate the environment and are toxic to humans. Recent data show that Brazil is one of the largest consumers of pesticides of the world, and may soon overtake the United States, and become the largest market for agrochemicals in the world (ANDEF, 2011).

Interestingly, the efficiency of essential oils used to control plant diseases have been reported recently by ROZWALKA et al. (2010) and PEREIRA et al. (2011; 2012). The authors investigated the action of EOs by electron microscope studies. In the same way, cow's whole milk has been used as alternate way to control powdery mildew (BETTIOL et al., 1999; STADNIK & BETTIOL, 2001). However there are few reports regarding the mode of action of these natural products in phytopathogens.

The goals of this research were to evaluate the potential and determine effective concentrations of cow's whole milk (CWM) and essential oils (EOs) in order to control powdery mildew on soybean plants and verify the effect of these natural products on ultrastructure of ***E*. *diffusa*** by scanning electron microscopy (SEM).

MATERIAL AND METHODS

Inoculation and plants maintenance under greenhouse

All of the experiments were conducted at Federal University of Lavras (UFLA) from October 2009 to August 2011. The inoculation technique used depended on the specific objectives of the experiment. In the greenhouse, trials aimed to evaluate the disease severity and effective concentrations. The plants were inoculated through direct contact between healthy and infected plants, using a rate of five healthy plants to one infected 30 days old plant (previously infected by spray inoculation with a 1.10^6 conidia mL^{-1} suspension in the first and second trifoliate leaf). For the SEM and LM trial, a spray inoculation was carried out using a suspension at 2.10^5 conidia mL^{-1}. Soybean plants Conquista cultivar (MG/BR 46) were grown in 3.0L pots containing substrate (expanded vermiculite, organic materials, macro and micronutrients), under greenhouse conditions during the whole experimental period.

Essential oils and cow's whole milk origin and composition

The cow's whole milk (CWM) used in these trials was obtained at the experimental farm at Department of Animal Science – UFLA. This milk was from antibiotic-free animals in order to avoid result interferences and was held in a refrigerated chamber at 4°C up to the application, performed 4 to 6 hours after milking. The average elemental composition of CWM used was 3.7% Fat; pH of 6.6; 3.5% of protein and 12.7% of total solids. The essential oils (EOs) were acquired from Professor Accorsi Medicinal Plants® (Piracicaba, São Paulo State, Brazil) to ensure a rigid pattern of cultural practices and quality. EOs of citronella [***Cymbopogon nardus*** (L.) W. Watson], lemongrass [***Cymbopogon citratus*** (DC. ex Nees) Stapf.], eucalyptus [***Eucalyptus globulus*** (Labill.)], cinnamon [***Cinnamomum zeylanicum*** (Blume)] and tea-tree [***Melaleuca alternifolia*** (Maiden. and Betche) Cheel] were used and the promising EOs were selected for the effective concentrations experiment.

EOs and CWM in the control of soybean powdery mildew severity under greenhouse and mechanism of action *in vivo*

EOs from all species previously cited were used at 1.0mL L^{-1} and the CWM was used at 100mL L^{-1}, being these concentrations already reported as effective against plant diseases (BETTIOL, et al., 1999; PEREIRA et. al., 2012). All treatments were diluted in distilled water and EOs treatments were added to Tween 20 (1.0mL L^{-1}) as an emulsifier. With the purpose of isolating the effect of the Tween 20, a treatment only composed of this substance was added at 1.0mL L^{-1} additionally and it was used a tebuconazole fungicide at 1.17mL L^{-1} as a standard control. At ten days after plant germination (DAG), the inoculation was performed with ***E. diffusa*** through direct contact between plants. At 20 and 50 days post inoculation (DPI) plants were sprayed until dripping with all treatments previously cited, by manual sprayer. The disease severity was evaluated five times through the experiment period at seven day intervals starting at 20DPI. All leaflets were evaluated in each experimental plot with a powdery mildew severity diagrammatic scale for common bean (AZEVEDO, 1997) adapted to soybean.

To elucidate the possible mechanism of action by EOs and CWM on the ***E. diffusa*** ultrastructure *in vivo*, the same treatments and concentrations from the first experiment were employed. This experiment used the detached leaves method and spray inoculation was performed at 10 DAG using an ***E. diffusa*** suspension. Hereafter, leaflets infected containing powdery mildew with uniform symptoms were collected at 10 DPI and stored in polystyrene tray (215mmx177mm) for subsequent treatments. Soon afterwards, the leaflets surface were sprayed with the same EOs, CWM and controls of the previous trial, by manual sprayer and incubated at 20°C±2°C for 24 hours with 12 hours of photoperiod. After incubation, 1cm^2 samples were collected from each leaflet in order to perform the SEM and LM analysis. For LM studies, conidia biomass were collected in triplicate with a soft brush in 1.0mL sterile water, centrifuged (3000rpm 5min^{-1}) and embedded in 2% agarose gel. The sample preparation for SEM and LM analysis were conducted according to the methodology for plant materials proposed by BOZZOLA & RUSSELL (1999), where LM's samples were embedded in Spurr's resin, cut by means of a Reichert-Jung Ultracut ultramicrotome, collected on a LM slide (10 to 15 serial 300-500µm sections), stained in 0.5% toluidine blue and viewed with a Zeiss Axio Observer Z.1 ligth microscope. The morphological SEM analysis was conducted in a scanning electron microscope LEO EVO 40XVP, at 20kV accelerating voltage and 12.0mm working distance.

Effective concentrations of EOs and CWM against powdery mildew of soybean

Aiming to improve the method efficiency and with the purpose of determining the most effective concentrations of the treatments selected in the previous trials, another experiment was conducted under greenhouse conditions. Products selected as most effective were EOs of citronella (CI), lemongrass (LG) and eucalyptus (EU) as well as the CWM. The EOs were used at 0.5mL L^{-1}, 1.0 mL L^{-1} and 2.0mL L^{-1} in distilled water added to Tween 20 at 1.0mL L^{-1}, and the CWM was used at 50mL L^{-1}, 100mL L^{-1} and 200mL L^{-1} in distilled water. The inoculation method, as well as the treatments application and disease severity evaluation were performed as described in the first experiment.

Experimental design and statistical analysis

All experiments were conducted in randomized blocs. In the trial conducted to evaluate the capacity of EOs and CWM to reduce the powdery mildew severity, three replicate plots containing two pots with two plants were used. In the mechanism of action *in vivo* experiment, six leaflets per treatment and four replicates were employed. Four replicates with plots composed by two pots with two plants were used in the trial aiming to determine effective concentrations of EOs and CWM. Data from the disease severity were transformed to the area under the progress curve for disease severity (AUPCDS), according to SHANER & FINNEY (1977). The control effectiveness percentage was calculated for each treatment according to ABBOTT, (1925). To precisely estimate the effective concentration, the most effective concentration to control was estimated by calculating the regression for each product selected as promising. The statistical analyses of the data were performed using the Sisvar (v. 4.5) statistical software. The qualitative means were grouped using the Scott-Knott test ($P \leq 0.05$) and regression graphs were generated for quantitative means.

RESULTS AND DISCUSSION

EOs and CWM in the control of soybean powdery mildew severity under greenhouse and mechanism of action *in vivo*

All treatments reduced the AUPCDS (Figure 1A) when compared to control treated with distilled water. EOs of CI, LG and EU provided the highest reductions in AUPCDS, 74.2%, 68.7% and 67.1%, respectively, which did not differ from the tebuconazole fungicide (FU) and CWM, these showed respectively 86.1% and 72.1% of reduction in the disease severity. Whereas the EO of cinnamon (CN) and tea-tree (TT) differed from the FU and CWM as they presented a reduction in AUPCDS of 57.3% and 44.9% respectively.

In the images generated by SEM (Figure 1B - I), it was possible to verify that in the control leaflets, treated with distilled water (Figure 1B), the morphology ultrastructure of the ***E. diffusa*** was intact and well developed. Abundant hypha, forming a dense fungal mycelium and spores layer, could be viewed in the epidermis of leaflets related to the control (Figure 1B), when compared to treatments composed by of EOs of CI (Figure 1C), LG (Figure 1D), CN (Figure 1E), EU (Figure 1F), TT (Figure 1G), and for treatments of CWM (Figure 1H) and FU (Figure 1I). EOs and FU caused a cell leakage and turgidity loss on ***E. diffusa*** conidia. It was reinforced by LM analysis which showed most clearly conidia damage for LG EO in contrast with non-affected water treatment (Figure 1 J and K respectively). A

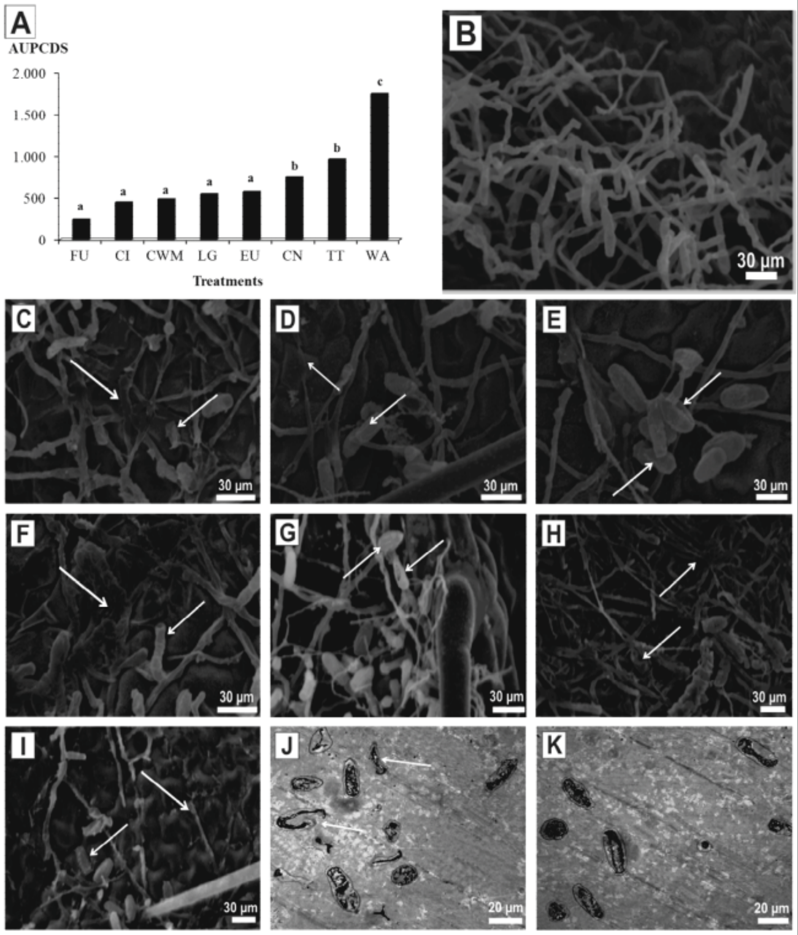

Figure 1 - A: Area under progress curve for powdery mildew severity (AUPCDS) on soybean plants treated by FU: fungicide tebuconazole (1.17mL L-1); CWM: cow's whole milk (100mL L-1). Essential oils (EOs) of CI: citronella; LG: lemongrass; EU: ***Eucalyptus globulus***; CN: cinnamon and TT: tea-tree all at 1.0mL L-1; WA: control sprayed with distilled water. Areas followed by same letter do not differ by the Scott-Knott test ($P<0.05$). B to I: Scanning electron micrographs of soybean leaflets infected by ***Erysiphe diffusa*** and treated by: WA (B); EOs (1.0mL L-1) of: CI, LG, CN, EU, TT; CWM at 100mL L-1; FU at 1.17mL L-1 in C; D; E; F; G; H and I respectively. J and K: light micrographs of the conidia treated with LG EO and water respectively. Arrows indicate the hypha leakage and loss of cell content in conidia.

similar effect was detected in the ***E. diffusa*** hyphae, with clear degeneration, turgidity loss and consequent reduction in mycelia growth in the epidermis of the leaflets exposed to EOs, CWM and FU. However, this was observed in most treatments, except in the leaflets treated with TT EO, which demonstrated damage only on conidia (Figure 1G).

Regarding the reduction of the AUPCDS, the result obtained for the CI's EO (74.2%), showed higher than the result related by MEDICE et al. (2007), which have demonstrated reduction in the Asian rust severity (***Phakopsora pachyrhyzi*** Syd. & P. Syd) on soybean of 53.18% in plants treated with CI EO. Likewise, the disease reduction using LG and TT EOs of this research (68.7% and 44.9% respectively), corroborated with the obtained by PEREIRA et al. (2012) who reported reductions in the incidence of coffee leaf rust of 67.7% and 55.4% using LG and TT EOs respectively and indicated a direct effect of these EOs.

This control by the EO can be attributed to both a direct effect of products as well as possible indirect mechanisms. Regarding the direct effect, the SEM analysis showed the damage to morphology of

E*. *diffusa (Figure 1B - I). This direct effect has already been previously observed by ROZWALKA et al. (2008) using the EO of LG, which showed fungitoxic activity on mycelia growth of ***Glomerella cingulata*** [(Stonemam) Spauld & Schrenk]. Additionally, the indirect effect control can also be attributed to the preventive action mode of EO as pointed out by PEREIRA et al. (2011) and demonstrated the involvement of induced resistance by LUCAS et al. (2012). This observation suggests a possible indirect control through induction of resistance in plants treated with EO. Similarly, for CWM, indirect effects may include induction of host resistance and stimulation of antagonistic microorganisms on leaf surfaces (BETTIOL et al., 2008). In this research, it is likely that effect occurred, resulting in increasing of plant protection, especially in parts of soybean plants where the powdery mildew had not yet established, culminating later in decreasing in the severity of the disease. Concerning indirect effects of CWM, STADNIK & BETTIOL (2001) observed the stimulation of microorganisms, especially bacteria, on the phylloplane of cucumber plants sprayed with milk. On the other hand, SUDISHA et al. (2011) worked with CWM to control the millet downy mildew disease and reported the elicitation of resistance and defense related enzymes by application of CWM. The occurrence of such events may have aggravated the control obtained by treatment where CWM was used in this research.

The damaging effects of CWM and its derivatives on powdery mildews structure have also been observed in other studies. CRISP et al. (2006) observed a similar effect when applying cow's milk whey on vine powdery mildew [***Erysiphe necator*** (Schwein.) Burrill], demonstrated the hypha collapse and conidia alteration within 24h after treatment. These authors associated these effects on the pathogen to the increased production of free radicals and also to the possible action of the lactoferrin an antimicrobial component of milk. Thereby, FERRANDINO & SMITH (2007) comparing the CWM, skim milk and sodium bicarbonate effect on the ability to reduce powdery mildew [***Podosphaera xanthii*** (Castagne) U. Braun & Shishkoff] on pumpkin [***Cucurbita pepo*** L. cv. Howden)], observed that treatments consisting of milk exceeded the sodium bicarbonate treatment, indicating that the fungal control by milk does not occur only due to direct effect, suggesting the interference of other mechanisms.

Previous studies in other pathosystems have reported the EOs effect on plant pathogens through SEM analysis. Recently, PEREIRA et al. (2011) reported the conidial germination inhibition of ***Cercospora coffeicola*** (Berk & Cooke) 8 hours after inoculation in coffee plants treated preventively, two days before pathogen inoculation. Similarly, MEDICE et al. (2007) made evident by SEM, a reduction in the size of ***P. pachyrhizi's*** uredia in soybean leaflets treated with the EO of CI. This observation reinforces a possible fungitoxic activity of the EOs in this research, also reported by ROZWALKA et al. (2010), and in the same way here observed by CWM, evidencing a curative control of the disease by these products.

Effective concentrations of OEs and CWM against powdery mildew of soybean

The powdery mildew severity showed a quadratic behavior as EOs and CWM concentrations increased (Figure 2A-D). The regression analysis allowed a better understanding of the difference between tested concentrations and its potential to control the disease, providing estimates for optimal and feasible concentrations. For all natural products tested, the disease severity correlated negatively with natural products concentration. For the CI, by adjusting the quadratic regression, the concentration was estimated to be 1.51mL L^{-1}, which provided the largest reduction in the AUPCDS, around 36.7% (Figure 2A). Similarly, in the LG treatment, the optimal concentration was estimated at 1.45mL L^{-1}, providing 34.6% of reduction in disease severity (Figure 2B). The optimal concentration was estimated at 1.49mL L^{-1} in the EU treatment (Figure 2C), representing 29.8% reduction in the disease severity. For EU treatment such as the CI and LG, it was observed that from the concentration of 1.0mL L^{-1}, there was stabilization in reducing the powdery mildew severity. For the CWM, an interesting fact was observed, showing that at concentrations of 50 and 100mL L^{-1}, there was a strong reduction in the AUPCDS, with subsequent stabilization, making it possible to infer that the optimal and cost-effective concentration to be applied are included in the interval between 70 and 200mL L^{-1} (Figure 2D), despite the optimal concentration found through the regression equation was 184mL L^{-1}, providing a disease severity reduction of 50.7%, demonstrating the most significant reduction of disease severity among the natural products.

Some authors have previously studied the different concentrations of cow milk on plants disease control. SUDISHA et al. (2011) used CWM at 5, 10, 20 and 30% to examine the preventive control against

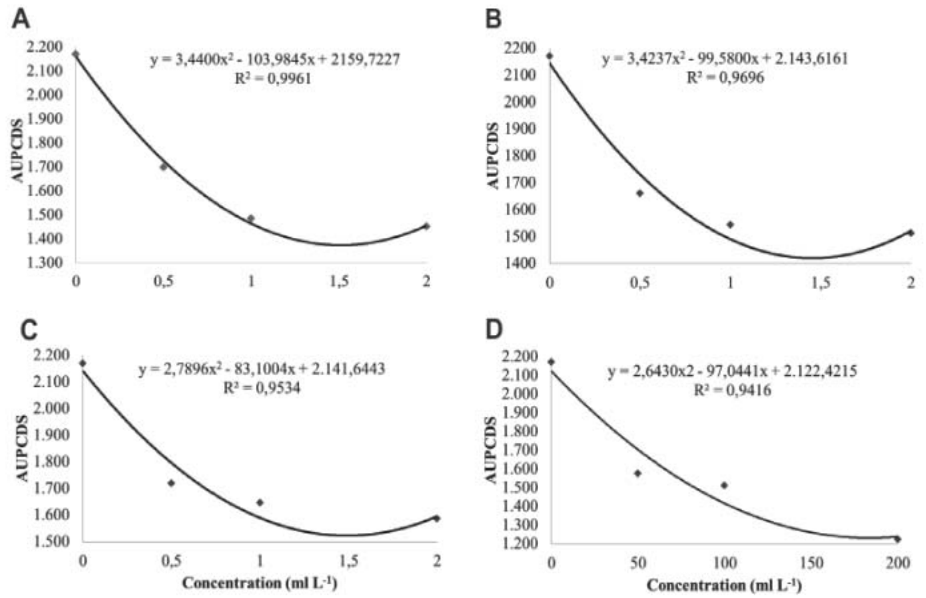

Figure 2 - Fitted quadratic regression for the area under the progress curve for disease severity (AUPCDS) related to concentrations (mL L-1) in plants treated with essential oils of the citronella (A); lemongrass (B); ***Eucalyptus globulus*** (C) and cow's whole milk (D) to control powdery mildew in soybean plants.

downy mildew disease [***Sclerospora graminicola*** (Sacc.) J. Schröt.] and observed the highest disease protection at 10% (100mL L^{-1}). According to the authors, the protection was due to the induction of the enzymes involved in resistance in plants. The results of this research corroborate with BETTIOL et al. (1999) that also observed a negative correlation between the infected leaf area and milk concentration sprayed on plants. In the same way, BETTIOL et al. (2008) demonstrated that the control of the powdery mildew on cucumber plants was directly proportional to the concentrations of whey derived from CWM. However in relation to EOs, this study reports one of the first studies involving the application of different concentrations and its effectiveness on plants disease.

It should be noted that the results obtained through SEM corroborate the data obtained in experiments conducted in the greenhouse, where the treatment with essential oil of tea-tree, cinnamon and eucalyptus promoted a smaller AUPCDS and lower activity under ***E. diffusa*** structures. Lastly, it can be argued that the EOs of CI and LG as well as CWM, are promising products to control powdery mildew on soybean especially in organic production fields. The results show the ability of those natural products to prevent the advance of soybean powdery mildew when applied directly to the plants. Nevertheless, complementary studies are needed to characterize the modes of action of EOs and CWM, to explore possible additive effects provided by the mixture of these products and to establish the ideal application frequency for the control of diseases.

CONCLUSION

The studied EOs of LG, CI and EU, as well as the CWM, provide a control similar to the tebuconazole fungicide, in the powdery mildew on soybean and are most effective in controlling disease than the EOs of CN and TT. The EOs of LG, CI, EU, CN and TT at a concentration of 1mL L^{-1}, as well as CWM (100mL L^{-1}) provide a direct effect on ***E. diffusa*** structures. The use of concentration twice higher than 1mL L^{-1} (0.1%) for EOs of LG, CI, EU, and 100mL L^{-1} for CWM, do not result in substantial increase in the control of the powdery mildew on soybean.

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