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Effect of essential oils on miceliogenic and carpogenic germination of *Sclerotiniasclerotiorum* (Lib.) de Bary

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Abstract

Background: *Sclerotiniasclerotiorum* is a pathogen that causes the disease known as Sclerotiniastem rot, present in several hosts, including crops of great economic expansion, such as soybeans, beans, potatoes, tomatoes, among others. In order to deepen the knowledge of phytopathogenic biology, an important tool is the evaluation of the viability of sclerodes through myceliogenic and carpogenic germination. **Objective:** Thus, the objective of this work was to verify the potential of 17 essential oils on myceliogenic and carpogenic germination of *S. sclerotiorum*. The viability of the sclerotia was performed through the induction of myceliogenic and carpogenic germination. The experimental design was a completely randomized with four replicates and 19 levels. The data were submitted to the analysis by Scott-Knott at 5% probability of error. **Results:** Essential oils of *Zingiberofficinale*, *Laurus nobilis*, *Artemisia vulgaris*, *Eucalyptus Citriodora*, *Thymus vulgaris*, *Citrus latifolia*, *Cyperusarticulatus*, *Cymbopogoncitratus*, *Eugenia uniflora*, *Schinus Terbinthifolius* and *Cinnamomumzeylanicum* were the best to control of myceliogenic germination andall the essential oils tested were efficient in controlling the formation and germination of apothecia. **Conclusion:** The 17 essential oils used in this study, expressed excellent results, since they were efficient when compared to the control treatment. It is believed that the fungicide action of these essential oils can be related to its major components, not ruling out the effect of the minority, causing an aggressive stimulus in the structures of the fungus, in this way, the cells broke their homeostatic balance and underwent a regressive process that can lead to cell death.

Key words: Essential oil, Myceliogenic germination, Carpogenic germination, Alternative control. Sclerotiniasclerotiorum.

INTRODUCTION

The fungus *Sclerotiniasclerotiorum* (Lib.) de Bary is a pathogen that causes the illness known as white mold or white rot of the stem. Your damages are severe and can affect several crops of great relevance in Brazil, causing irreversible losses, becoming an important factor in production (Botelho *et al*., 2013).

With the advancement of disease sclerotia are formed, these return to the soil with cultural remains and are responsible for the survival of the phytopathogen (Garcia *et al*., 2012). Through sclerodes, myceliogenic germination can occur, where hyphae will contaminate the plant, and/or carpogenic germination, a precursor of apothecia responsible for the production and release of millions of ascospores in the infected area (Görgen, 2009).

Therefore, preventive measures are necessary and must be taken together to avoid the entry of the pathogen into new areas, since once it is present, it isdifficult to eradicate it (Barbosa and Gonzaga, 2012). Generally, the farmers use chemical fungicides, however, because it is a soil fungus, with resistance structure, the use of fungicides, besides expensive, does not present satisfactory results.

According to De Souza Zanella *et al*. (2015), the demand for pesticide-free plants is increasing, motivating the search for new measures to protect plants against diseases. Therefore, research related to the fungicide potential of natural substances is pertinent, since it may contribute to the alternative control of plant diseases of agricultural importance, as well as to reduce environmental contamination and human health problems caused by the use of synthetic fungicides.

Studies indicate that substances produced by some plants act as fungistatic or fungicidal agents (Antunes and Cavacob, 2010; Silva *et al*., 2009). Essential oils are secondary metabolites, also known as natural antifungals. These have a complex and variable chemical composition among species, age, place of cultivation, parts of the plant, edaphoclimatic factors.These substances guarantee to the plants adaptive advantages in the environment in which they are inserted (Oussalah *et al*., 2007; Miranda *et al*.2016).

These oils can provoke irreversible damage to the cell membrane of the cells, leaving them soluble and with coarse fractures exposing the cellular content, including the nucleus leading to cell death (Silva *et al*., 2009), or may also favor the growth and development of pathogens. Therefore, the goal of this work was to evaluate the effect of different essential oils on the myceliogenic and carpogenic germination of *Sclerotiniasclerotiorum* (lib.) de Bary.

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MATERIAL AND METHODS

2.1. Obtaining, multiplication and maintenance of inoculum:

Two experiments were carried out, the first to evaluate the myceliogenic germination and the second to evaluate the carpogenic germination of *S. sclerotiorum*. The experiments were conducted at the Phytopathology Laboratory of the Federal Technological University of Paraná (UTFPR), from March 2014 to May 2015.

The isolates of *S. sclerotiorum* were obtained from the pure colony of the fungi collection in the Phytopathology laboratory of the university itself. The sclerotia were produced from the mycelial discs of the fungus in laminar flow, to Petri® dishes sterilized with PDA (potato, dextrose and agar) culture medium properly autoclaved. The plates were incubated at 19° C, in photoperiod for 7 days. After this period, the formed sclerotia were removed from the plates, and stored at 5° C until their use in the experiments.

2.2. Experimental design and data analysis:

The experimental design was a completely randomized design with four replications for the two evaluations (carpogenic and myceliogenic). For both experiments the data were submitted to the analysis of averages by the Scott-Knott test at 5% probability of error.For comparison of the averages was used the software ASSISTAT 7.6 beta. All the results were submitted to normality test and had to be transformed by the X+C equation. For the myceliogenic germination, in addition to the ASSISTAT, the software Microsoft®Excel 2010 was also used to perform the linear regressions.

2.3. Essential oils of species:

Essential oils used for the study were: *Zingiberofficinale*Roscoe, *Laurus nobilis*L., *Citrus reticulate* Blanco, *Artemisia vulgaris* L., *Eucalyptus Citriodora*Hook,, *Syzygiumaromaticum*(L.) Merrill & Perry, *Melaleuca alternifólia*Cheel, *Thymus vulgaris* (L.), *Citrus sinensis*L, *Citrus latifolia* Tanaka,, *Psidium guajava*Linn,, *Cyperusarticulatus*L., *Cymbopogon citrates* (DC) Stapf, *Eugenia uniflora*L, *Casearíasylvestris*Sw,, *Schinusterbinthifolius*Raddi, and*Cinnamomumzeylanicum* Blum, obtained from the hydrodistillation process, coming from the company "Cacalia - ProdutosNaturais".

These oils were used for the two viability experiments of myceliogenic and carpogenic germination of *S. sclerotiorum* sclerotia. These oils were purified with membrane type filter for 0.45 µm pore size syringes; 30 mm outer diameter; 4.3 cm^2 filtration area; 100mm processing volume; volume retained after filtration of less than 100 µL and external polypropylene material, reserved in Eppendorf® micro tubes. For myceliogenic germination, 5 sclerotia were immersed in 15 µl + 2 ml of water and 1 drop of Tween for 2 minutes. For carpogenic germination, 60 sclerotia in 30 µl + 2 ml of water and 1 drop of Tween for 2 minutes. It should be noted that the dose (15 µl) in the concentration (100%) chosen were based on results from other works conducted in the phytopathology laboratory, as well as other researches.

2.4. Myceliogenic germination under different essential oils:

Evaluation of myceliogenic germination was carried out in PDA (potato, dextrose and agar) culture medium. The sclerotia were previously disinfested in 70% alcohol for 1 minute, 1% sodium hypochlorite for 1 minute, washed in autoclaved water for 1 minute and air dried in an aseptic environment inside a laminar flow chamber. The experiment was composed of 4 replicates (5 sclerotia) with 19 treatments, in each Petri® dish, containing a culture medium, five sclerotia with a size of 1 to 2 mm, duly treated in 15 µl + 2 ml of water + 1 drop of Tween (for 2 minutes), were placed to germinate. Incubation was performed in a BOD-type growth chamber at 22 ± 3° C and 12-hour photoperiod and evaluations were performed at 24, 48, 72 and 96 hours after incubation. A stereoscopic microscope was used in which the appearance of characteristic hyphae of the fungus was observed. In each evaluation the number of sclerotia that had germination was determined.

2.5. Carpogenic germination under different essential oils:

Evaluation of carpogenic germination was carried out in Dystroferric Red Nitosol soil, typical of clayey texture (Bheriva, *et al*., 2008), collected in Dois Vizinhos - PR on university campus. After the collection, the soil was autoclaved at a temperature of 121º C and a working pressure of 1.2 Kgf/cm². The time of sterilization of the soil in an autoclave was 1 hour by autoclaving, and it was carried out for three days under a 24 hour interval between them.

The experiment was composed of 4 replicates (5 sclerotia) with 19 treatments, the samples of 200g of soil were placed in Gerbox® boxes (11cm x 11cm x 3.5cm). The sclerotia corresponding to each treatment (60 sclerotia) were treated by different oils, using 30 µL of these in 2 ml of water and one drop of Tween, and two control treatments, one with water and Tween and another only with water. Ten sclerotia of the fungus were buried to the depth of approximately 0.3 cm. In sequence, a 6.0 mm water slide was applied per box, leaving soil moisture close to field capacity through the use of irrigation. The boxes were incubated at 18° C under a photoperiod of 12 h light and 12 h dark for a period of 40 days. Feasibility assessments were carried out in two ways, the first, counting if the apothecia were issued and the second the number of stipes and apothecia formed by Gerbox® box. The first evaluation was performed after the first stipe was issued, until the completion of 40 days of incubation. Sclerodes that germinated by the production of mycelium and subsequent production of sclerotia were considered viable.

3. Results:

3.1. Myceliogenic germination:

The results of the myceliogenic germination can be verified in Table 1, which shows the average of myceliogenic germination of the sclerotia in the different evaluation times, times of 24, 48, 72 and 96 hours. Data were submitted to the Shapiro-Wilk normality test and then to the Scott-Knott test at 5% error probability.

In the first evaluation (24 hours of incubation) there was no germination in the treatments, and there was no statistical difference between them. In the 48 h period, the results showed that the two witnesses differed from the other treatments with higher germination values. The oils of *Citrus reticulata, Melaleuca alternifolia, Citrus sinensis, Psidium guajava, Cyperusarticulatus, Eugenia uniflora, CasearíasylvestriseSchinusTerbinthifolius*also began their germination in this period and differed statistically from the other treatments. In the third evaluation of 72 hours, all previously mentioned treatments continued to germinate, with the exception of *SchinusTerbinthifolius* and *Cyperusarticulatus* that maintained the germination stabilized. The oils of *Zingiberofficinale, Laurus nobilis, Artemisia vulgaris,* and *Syzygiumaromaticum* began to germinate only after this period. In the last evaluation of 96 h still in Table 1, it is observed that the two witnesses did not differ among themselves, which is a satisfactory result, since it demonstrates that the witness that owns the Tween additive is not responsible for inhibiting the myceliogenic germination of the sclerotia, thus proving the real effect of essential oils. Witnesses differed from the other 17 treatments, proving that even the values of the highest germination treatments were lower in relation to the germination of the witnesses. The low or 100% inhibition valuesof myceliogenic germination were verified in the treatments with oils of *Zingiber officinale, Laurus nobilis, Artemisia vulgaris, Eucalyptus Citriodora, Thymus vulgaris, Citrus latifolia, Cyperusarticulatus, Cymbopogoncitratus, Eugenia uniflora, Schinus Terbinthifolius, Cinnamomum zeylanicum*, these did not differentiate between themselves, but they differed from the other treatmentsand demonstrate a positive potential in the control of myceliogenic germination of *S. sclerotinia*.

Table 1: Average of myceliogenic germination of sclerotia at different times. Dois Vizinhos-PR, 2017.

Treatments	24 Hours	48 Hours	72 Hours	96 Hours
Witness	0 a	40 a	60 a	90 a
Witness with Tween	0 a	35 a	50 a	100 a
Zingiberofficinale	0 a	0 c	10 b	15 c
Laurus nobilis	0 a	0 c	10 b	15 c
Citrus reticulata	0 a	20 b	40 a	60 b
Artemisia vulgaris	0 a	0 c	20 b	30 c
Eucalyptus Citriodora	0 a	0 c	0 b	0 c
Syzygiumaromaticum	0 a	0 c	30 a	40 b
Melaleuca alternifolia	0 a	20 b	30 a	40 b

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Thymus vulgaris	0 a	0 c	0 b	0 c
Citrus sinensis	0 a	20 b	35 a	40 b
Citrus latifolia	0 a	0 c	0 b	0 c
Psidium guajava	0 a	20 b	35 a	45 b
Cyperusarticulatus	0 a	5 c	5 b	5 c
Cymbopogoncitratus	0 a	0 c	0 b	0 c
Eugenia uniflora	0 a	10 c	20 b	20 c
Casearíasylvestris	0 a	20 b	35 a	40 b
SchinusTerbinthifolius	0 a	10 c	10 b	10 c
Cinnamomumzeylanicum	0 a	0 c	0 b	0 c
CV%	0.00	24.31	31.26	34.82

Averages followed by distinct letters differ themselves by the Scott-Knott test at 5% probability of error.

3.1.1. Discussion:

According to a study by Young and Werner (2012), the petals of rapeseed flowers are the main route for the entry of the pathogen in the plant, so the period in which the plant will bloom becomes an important factor for the disease to settle in crop. Therefore, even if the essential oils of *Citrus reticulata*, *Syzygiumaromaticum*, *Melaleuca alternifolia*, *Citrus sinensi*, *Psidium guajava* and *Casearíasylvestris* germinated their sclerotia, they did not match the controls and the minimum germination suppression was 40%, which is a great value compared with the witnesses.

Similar results were reported in work of Pansera *et al*. (2012), where the use of essential oils of *Cymbopogoncitratus*, *Salvia officinalis* and *Baccharistrimera*, had a fungitoxic effect on the pathogen *S. sclerotiorum*. These authors observed that the plant extracts of *Cymbopogomcitratus*, *Schinusmolle*, *Schinus terebinthifolius*, *Salvia officinalis* and *Bacharistrimera*, when tested against the *S. sclerotiorum* fungus did not present control, and they attributed these results to a possible inadequacy of the doses used. However, Costa *et al*. (2011) showed morphological changes in the fungal mycelium when treated with a concentration of 0.15% of clove essential oil. Bajpai *et al*. (2007), studying the fungitoxic effect of *Metasequoiaglyptostroboides*Mikiex Hu essential oil, observed that this essential oil at the concentration of 1000µg mL-1 caused a 56% mycelial inhibition on *S. sclerotiorum* demonstrating the fungitoxic activity of this oil.

The potential of the essential oil of *Schinusterebenthifolia*, which at the end of the experiment demonstrated 90% inhibition of myceliogenic germination, was observed in this work. This data corroborates with studies by Scrivanti *et al*. (2003), which confirmed the antifungal activity of this oil, with positive results for the tests with the fungi of *Aspergillus niger*, *A. flavus*, *A. parasiticus*, *A. fumigatus* and *Trichoderma spp*.

Table 2 shows the regression equations for the 19 treatments. The germination average presented the best fit for the linear model of the *Citrus reticulata* essential oil for the germination time. However, the treatments of *Eucalyptus Citriodora*, *Thymus vulgaris*, *Citrus latifolia*, *Cymbopogoncitratus* and *Cinnamomumzeylanicum* showed no germination, and it was not possible to adjust the model because there was no regression.

Table 2: Linear regression equations of myceliogenic germination of sclerotia for the 19 treatments. Dois Vizinhos-PR, 2017.

Treatments	ŷ	R^2 (%)
Witness	y = 1,45x - 1,25	98,36
Witness with Tween	y = 1,575x - 1,625	95,99
Zingiberofficinale	y = 0,275x - 0,375	89,63
Laurus nobilis	y = 0,275x - 0,375	89,63
Citrus reticulata	y = x - 1	100
Artemisia vulgaris	y = 0,55x - 0,75	89,63
Eucalyptus Citriodora	y = 0	0
Syzygiumaromaticum	y = 0,75x - 1	88,24
Melaleuca alternifolia	y = 0,65x - 0,5	96,57
Thymus vulgaris	y = 0	0
Citrus sinensis	y = 0,75x - 0,625	97,83
Citrus latifolia	y = 0	0
Psidium guajava	y = 0,75x - 0,625	97,83
Cyperusarticulatus	y = 0,075x	60
Cymbopogoncitratus	y = 0	0
Eugenia uniflora	y = 0,35x - 0,25	89,09
Casearíasylvestris	y = 0,675x - 0,5	94,06
SchinusTerbinthifolius	y = 0,15x	60
Cinnamomumzeylanicum	y = 0	0

3.2. Carpogenic germination:

In Table 3, for the variable germination of stipe and total emission of stipes, it was possible to observe that the two witnesses did not differ statistically from each other, but they differed from the other treatments. With the exception of the *Citrus sinensis* essential oil, the other treatments did not differentiate between them, obtaining low and null results of stipe germination. For the germination of apothecia and for total emission of apothecia, both witnesses were differentiated among themselves and between the other treatments. And all the other treatments did not differ from each other.

Table 3: Percentage of *Sclerotiniasclerotiorum* sclerotia germinated at 40 days after application of essential oils; Average number of stipes by sclerotia; Average number of apothecia per sclerodium; And the relation between stipes and formed apothecia. Dois Vizinhos, UTFPR, 2017.

Treatments	Stipe germination	Total emission of stipe	Apothecia germination	Total emission of apothecia
Witness	11.75 a	21.50 a	10.25 a	18.50 a
Witness with Tween	11.00 a	24.75 a	7.25 b	12.50 b
Zingiberofficinale	1.50 c	3.50 c	0.00 c	0.00 c
Laurus nobilis	2.75 c	6.50 c	0.00 c	0.00 c
Citrus reticulata	1.25 c	2.25 c	0.00 c	0.00 c
Artemisia vulgaris	1.75 c	2.75 c	0.50 c	1.00 c
Eucalyptus Citriodora	1.50 c	2.50 c	0.00 c	0.00 c
Syzygiumaromaticum	1.00 c	3.50 c	0.00 c	0.00 c
Melaleuca alternifolia	1.25 c	3.25 c	0.25 c	0.25 c
Thymus vulgaris	3.25 c	7.00 c	0.50 c	0.75 c
Citrus sinensis	8.25 b	15.50 b	3.00 c	5.75 c
Citrus latifolia	2.25 c	6.00 c	1.00 c	2.25 c
Psidium guajava	0.00 c	0.00 c	0.00 c	0.00 c
Cyperusarticulatus	0.00 c	0.00 c	0.00 c	0.00 c
Cymbopogoncitratus	0.25 c	0.25 c	0.00 c	0.00 c

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Eugenia uniflora	0.00 c	0.00 c	0.00 c	0.00 c
Casearíasylvestris	0.00 c	0.00c	0.00 c	0.00 c
SchinusTerbinthifolius	0.00 c	0.00 c	0.00 c	0.00 c
Cinnamomumzeylanicum	0.00 c	0.00 c	0.00 c	0.00c
CV%	37.21	68.55	54.68	78.33

Averages followed by the same letter in the column do not differ from each other by the Scott-Knott test at 5% probability.

3.2.1. Discussion:

Data obtained by De Souza Zanella *et al*. (2015), observed that the *Annona cacan* extract, at 50 days, inhibited the emission of the number of apothecia in relation to the witness and the extracts of *Annona dioica* and *Annona coriacea*.

The results found in this work with essential oil of *Zingiberofficinale*, demonstrate the suppression of 100% germination and total emission of apothecia and 85% in myceliogenic germination, data different from those found by Rodrigues *et al*. (2007), who observed that the aqueous extract of *Zingiberofficinale* inhibited in almost 30% the production of sclerotia in the concentration of 25%. This difference can be justified by the fact that essential oil has a complex structure, and can sometimes be composed of more than a hundred chemical components, differing from the aqueous extract, as well as the oil used in this work was not diluted, using 100% of its concentration.

According to works by Chong *et al*. (1997), the essential oil of *Syzygiumaromaticum* exhibits as major component eugenol and researches point to this compound as a potential fungicide (Delespaul, 2000), corroborating with the data found in this work, since this same oil had effect fungicide, not allowing the formation and germination of apothecia.

In a paper by Zambonelli *et al*. (1996), using *Thymus vulgaris* essential oil at 800ppm, observed a reduction in the mycelial growth of *Colletotrichumlindemuthianum* and *Pythium ultimum*, causing hyphae deterioration and extravasation of the cellular cytoplasm. In this work, this oil showed potential in the suppression of carpogenic germination.

Mazaro *et al*. (2008) reports that the diversity of secondary metabolites present in *Eugenia uniflora* may present potential for the use compounds of this plant in agriculture, for the activation of defense routes, with activation of metabolites, and may present capacity for alternative control of pathogens in plants.

Conclusion:

The *Zingiber officinale*, *Laurus nobilis*, *Artemisia vulgaris*, *Eucalyptus Citriodora*, *Thymus vulgaris*, *Citrus latifolia*, *Cyperusarticulatus*, *Cymbopogoncitratus*, *Eugenia uniflora*, *Schinus Terbinthifolius* and *Cinnamomumzeylanicum* essential oils were the best in myceliogenic germination control and all the essential oils tested were efficient in the control of the formation and germination of apothecia. Thus, it is believed that the fungicide action of these essential oils may be linked to its major components, not ruling out the effect of the minority, causing an aggressive stimulus in the structures of the fungus. In this way the oils broke the fungus cells, destabilizing their homeostatic balance, triggering a regressive process that may have led to cell death. According to the toxicity results of the oils in relation to the fungus, there is an indication of good perspectives for their experimental use to control them. Different concentrations should be tested in vitro. The control of *S. sclerotiorum* in greenhouse and in the field should also be investigated, since biotic and abiotic factors can reduce the antifungal activity of the oils in an uncontrolled environment. The reduction of the number of stipes and consequently apothecia, through the essential oils, demonstrates a delaying action in the fungus sexual reproduction, causing a possible reduction or inhibition in the speed of infection of the same in the plants, since this emits their ascospores through this ascocarp.

The essential oils action over the fungus demonstrates an innovative character, since it preaches the alternative control of this disease, without the use of chemical fungicides. In addition, all oils are derived from plants with ease of production and fast-growing capacity, thus facilitating the extraction of the essential oil from them.

Future work:

The results obtained in this study can be used as a basis for future works, since the 17 essential oils tested showed excellent results, that should be better explored with different dosages, as well gas chromatography to know the main components. More specialized studies about the direct effect on hyphae should also be performed in order to understand the mechanism of action of these oils on *Sclerotiniasclerotiorum*.

Because it has a resistance structure that can last for a long time in the soil, control by fungicides becomes an expensive and not always effective method. Later studies will also investigate the *in vivo* behavior of these oils in plants attacked by fungus, so that products derived from them can be used as an alternative to synthetic fungicides, that cause so much contamination damage to the soil, plants themselves and especially humans.In addition, tests with essential oils may lead to discovery of more efficient products in the control of plants fungi and the reduction of the resistance of fungi to fungicides, since after studies, innovative molecules to control these pathogens can coming to market. Studies with essential oils in other pathogens should also be performed, so that their potential for control in other phytopathogenic diseases is analyzed.

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Highlights and contribution

- *The action of the essential oils on the fungus Sclerotinia sclerotiorum demonstrates innovative character, as it preaches the alternative control of this disease, without the use of chemical fungicides. In addition, all the oils used in the experiment are derived from plants with ease of production and rapid growth capacity, thus facilitating the extraction of the essential oil from them.*

- *To the world, through the results obtained in this study, new work could be done to prove the mode of action of these essential oils tested, with good perspectives to control the fungus under study, especially in organic crops, being more efficient than fungicides and not causing toxicity to mammals. The essential oils of Zingiber officinale, Laurus nobilis, Artemisia vulgaris, Eucalyptus citriodora, Thymus vulgaris, Citrus latifolia, Cyperus articulatus, Cymbopo goncitratus, Eugenia uniflora, Schinus terbinthifolius and Cinnamomum zeylanicum were efficient to control the fungus in its stage of myceliogenic germination and all the 17 essential oils tested were efficient in the carpogenic germination controlling the formation and germination of apothecia.*

- *According to the toxicity results of the essential oils in relation to the fungus, there is good prospects for their experimental use to control them. Higher concentrations should be tested in vitro as well as lower concentrations. The control of S. sclerotiorum in greenhouse and in the field should also be investigated, since biotic and abiotic factors can reduce the antifungal activity of the oils in an uncontrolled environment.*