

RESEARCH ARTICLE

Antifungal activity of essential oils on mycelial growth of *Fusarium oxysporum* and *Botrytis cinerea*

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ABSTRACT

In vitro study of the effect of different volumes of twelve essential oils on the mycelial growth of economically significant phytopathogenic fungi (*Fusarium oxysporum* and *Botrytis cinerea*) and it was compared to the effect of a fungicide. The antifungal activity of essential oils is decreased with the duration of incubation and it differs depending on the type of phytopathogenic fungus and the applied volume. The most effective antifungal effect on both tested fungi was in the essential oil of thyme, with lowest values of IC₅₀ while the weakest effect was in essential oils of eucalyptus and lemon, with the highest values of IC₅₀. Certain essential oils, when applied in certain volumes, had the same or even better effect on the inhibition of the growth of mycelium when compared to the tested fungicides.

Keywords: Essential oils; Phytopathogenic fungi; Mycelium growth; IC₅₀ values; Fungicides

INTRODUCTION

Plants pathogens cause deceases of plants in the field and warehouses and decrease the yield and quality of crop which eventually leads to great economic losses. The causes of plant deceases on the global level still cause losses of between 10 and 16 percent (Chakraborty and Newton, 2011). According to the opinion of the international community, *Botrytis cinerea* Pers is in the first place, with *Fusarium oxysporum* Schl. in the fifth place of economically most significant plants pathogens (Dean et al., 2012). The ability to adjust to various agroclimatic conditions allows the species of *Fusarium* to spread all over the world (Ćosić et al., 2004). *B. cinerea* adjusts its enzymes, depending on the specificity of the host and has the ability to adjust to the host it is infecting (Fillinger and Elad, 2016). The most dangerous fungal products are mycotoxins, secondary metabolites, which have a toxic effect on the organisms of people and animals. The exposure of population to mycotoxins is a growing problem in African countries due to everyday consumption of the infected corn (Degraeve et al., 2016).

The causes of plant deceases are still most effectively fought by the use of synthetic fungicides. However, the excessive, frequent and inappropriate use of fungicides

has caused many problems in their control, especially due to the development of resistant pathogen populations (da Cruz Cabral et al., 2013), through the residue accumulation in food above the permitted limit (Ogah et al., 2016), environmental pollution and negative effects on human health. All of these have led to an increased pressure of the public to decrease the use of synthetic fungicides (Bhagwat and Datar, 2014) and to conduct many studies in the search for the new and alternative agrochemicals (Combrinck et al., 2011).

Essential oils (EO) are secondary plant metabolites which often possess antimicrobial features and, as such, have an important role in the defence of the plants (Hyltdgaard et al., 2012). Due to their antifungal, antiviral and insecticidal characteristics, they are a desirable source of alternative methods in the protection of plants (Bassolé and Juliani, 2012). EO can also have a significant role in prolonging the food shelf-life and overcoming losses caused by storing agricultural products (Farzaneh et al., 2015; Prakash et al., 2015). They mostly consist of monoterpenes and sesquiterpenes and their oxygenated derivatives, such as alcohol, ketones, acids, phenols, ethers, esters, etc. (Fornari et al., 2012). They may contain more than 60 different ingredients, two or three of which are present in high concentration while others are

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present only in traces (Bakkali et al., 2008). Their antimicrobial effect depends on all chemical components (Queiroga et al., 2007) and the interactions among these components may lead to antagonistic, additive or synergistic effects (Bassolé and Juliani, 2012).

As the biological activity of essential oils depends on the type of plants and their chemical composition, applied concentrations, that is, volumes, environmental conditions, agro-technical practice (Ćosić et al., 2014), manner of application (Suhr and Nielsen, 2003) and other factors, there are studies being conducted on antifungal effect of essential oils (Palfi et al., 2018; Li et al., 2017; Pedrotti et al., 2017; Sharma et al., 2017; Nosrati et al., 2011; Ćosić et al., 2010; Huang et al., 2010) in order to find the most effective essential oils or their combinations.

The purpose of this research was to test the effect of twelve commercial essential oils in the *in vitro* conditions on the growth of mycelium *Fusarium oxysporum* and *Botrytis cinerea* and compare them with the effects of commercial fungicides but also IC_{50} for every essential oil.

MATERIAL AND METHODS

Essential oils

Twelve commercial essential oils were used in the study (*in vitro*): tea tree (*Melaleuca alternifolia* L.), anise (*Pimpinella anisum* L.), lemon (*Citrus limon* L.), pepper mint (*Mentha × piperita* L.), fennel (*Foeniculum vulgare* Mill.), basil (*Ocimum basilicum* L.), eucalyptus (*Eucalyptus globulus* L.), rosemary (*Rosmarinus officinalis* L.), true lavender (*Lavandula angustifolia* Mills.), thyme (*Thymus vulgaris* L.), clove (*Eugenia caryophyllata* L. Merr. & Perry) and sage (*Salvia officinalis* L.).

Essential oils were produced in Pranarôm International Ltd. (Belgium) except the essential oil of anise which was produced in Kemig Ltd., Sesvete-Soblinec, Croatia.

The study was conducted with eight volumes of essential oils: 3, 5, 7, 9, 15, 30, 50 and 70 μ L/10 mL potato dextrose agar (PDA). PDA substrates, produced by Merck KGaA, (Darmstadt, Germany), were used in the experiment, as well as the auxiliary agent Polysorbatum 80 (Tween 80) produced in Kemig Ltd., Donja Zelina, Croatia. The concentration of Tween 80 was 0.05%. Distilled water was used in the control variable of the experiment instead of essential oils.

Fungicides

The effect of the fungicide Prosaro 250 EC (prothioconazole + tebuconazole) was tested for the phytopathogenic fungus *Fusarium oxysporum*, and for *Botrytis cinerea*, the effect of the fungicide Switch 62.5 WG (cyprodinil + fludioxonil)

was tested. The amounts of fungicides were determined according to the manufacturer's instructions for each used fungicide. The Prosaro 250 EC fungicide was used in four quantities (75 mL/20 L H₂O, 75 mL/40 L H₂O, 100 mL/20 L H₂O, 100 mL/40 L H₂O) while Switch 62,5 WG fungicide was used in two quantities (60 g/100 L H₂O, 100 g/100 L H₂O). Instead of water, the same amount of prepared PDA substrate was used.

Phytopathogenic fungi

Single spore isolates of *F. oxysporum* i *B. cinerea* were obtained from the culture collection of Faculty of Agriculture in Osijek, Osijek, Croatia.

Procedure

10 mL of the PDA substrate was poured into each Petri dish (diameter 90 mm), and auxiliary agent Tween 80 was added as well as the previously determined volumes of essential oils. Discs of mycelium of eight-day old phytopathogenic fungi (diameter 4 mm) were placed in the middle of the Petri dish. The experiment was done for each volume of essential oils, fungicides and for the control variable in four replicates. After the inoculation, the Petri dishes were placed into an incubator, at the temperature of 20 °C and under the light regime of 12 h of light/12 h of dark.

The fourth and the eighth day since the beginning of the incubations, the diameter of the growth of mycelium of each pathogenic fungus was measured and expressed in mm.

Statistical data interpretation

Data analysis and graphs were made using the GraphPad Prism Version 7 program (GraphPad Prism, <https://www.graphpad.com/scientific-software/prism/>). The data are expressed as an arithmetic mean, standard deviation and 95 percent confidence interval for the IC_{50} parameter. IC_{50} was calculated as a concentration of the tested compound which decreases the mycelial growth by half between the base and maximum response by using the subroutine in the GraphPad Prism program.

Other data are expressed as mean and standard deviation. P value is the result of comparison with control using Dunnett's multiple comparison test, which prevents false positive results (Type I error). P values less or equal 0.05 were considered statistically significant.

RESULTS

The effect of the used fungicide doses

The ratio between the mycelial growth diameters for the phytopathogenic fungi *Fusarium oxysporum* and *Botrytis cinerea*

was compared and expressed in millimetres, in variants of fungicide and control variable without the use of fungicide was compared.

The results, shown in Table 1, show that the fungicide Prosaro 250 EC in all used doses, completely (100%) counteracts the growth of *F. oxysporum* fungus on the fourth and eighth day after the mycelial inoculation. In the control variable the mycelial growth of 68 ± 0.0 mm four days after the inoculation was measured, while on the eighth day the diameter of the mycelial growth was 90 ± 0.0 mm.

Fungicide Switch 62.5 WG completely inhibited the growth of *B. cinerea* fungus on the fourth day, while on the eighth day after the inoculation, the mycelial growth was 4 ± 3.1 mm, that is, 23 ± 2.9 mm, depending on the used dosage. In the control variable, the maximum mycelial growth was measured at 90 ± 0.0 mm already on the fourth day after the inoculation.

Ratio between the used volumes of essential oils and the diameter of the mycelial growth of *Fusarium oxysporum* and *Botrytis cinerea*

On the fourth day after the inoculation of fungus *F. oxysporum*, the weakest effect was determined in the

Table 1: The effect of applied fungicide doses (g/L or mL/L) on *Fusarium oxysporum* and *Botrytis cinerea* (mm) mycelium growth in comparison to the control

| Phytopathogenic fungus | Fungicide | Dose | Diameter of the mycelial growth (mm) and P value (vs Control) | |
|---------------------------|----------------|------------------------------|---|-----------------|
| | | | Day 4 | Day 8 |
| | | | | |
| <i>Fusarium oxysporum</i> | Prosaro 250 EC | 75 mL/20 L H ₂ O | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| | | 75 mL/40 L H ₂ O | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| | | 100 mL/20 L H ₂ O | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| | | 100 mL/40 L H ₂ O | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| | | Control | 68±0.0 | 90±0.0 |
| <i>Botrytis cinerea</i> | Switch 62.5 WG | 60 g/100 L H ₂ O | 0±0.0 (<0.001) | 24±3.1 (<0.001) |
| | | 100 g/100 L H ₂ O | 0±0.0 (<0.001) | 23±2.9 (<0.001) |
| | | Control | 90±0.0 | 90±0.0 |

(a) Results are expressed as mean±SD (n=4).

(b) Data in parentheses are P values of Dunnett test (comparison with control).

Table 2: The relationship between the applied volumes of essential oils (µL/10 mL PDA) and diameter (mm) of *Fusarium oxysporum* mycelium in which total mycelium inhibition was observed - fourth days

| Essential oils | Volumes (µL/10 mL PDA) and P value (vs Control) | | | | | | | |
|-------------------------------|---|--------------------|--------------------|--------------------|---------------------|--------------------|---------------------|--------------------|
| | 3 | 5 | 7 | 9 | 15 | 30 | 50 | 70 |
| <i>Melaleuca alternifolia</i> | 59±2.4 (0.005) | 60±4.0 (0.015) | 52±3.1 (<0.001) | 52±6.8 (<0.001) | 45±4.2 (<0.001) | 9±2.7 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Pimpinella anisum</i> | 60±3.3 (<0.001) | 55±3.7 (<0.001) | 30±1.7 (<0.001) | 35±2.1 (<0.001) | 8±2.4 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Citrus limon</i> | 51±1.7 (<0.001) | 51±2.5 (<0.001) | 52±1.7 (<0.001) | 49±3.3 (<0.001) | 53±2.5 (<0.001) | 32±1.8 (<0.001) | 27±2.4 (<0.001) | 23±3.4 (<0.001) |
| <i>Mentha x piperita</i> | 58±4.0 (<0.001) | 60±6.0 (<0.001) | 49±3.4 (<0.001) | 47±9.9 (<0.001) | 36±3.8 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Foeniculum vulgare</i> | 42±2.6 (<0.001) | 49±2.9 (<0.001) | 31±0.8 (<0.001) | 30±1.7 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Ocimum basilicum</i> | 55±0.8 (<0.001) | 56±2.6 (<0.001) | 61±1.3 (0.010) | 53±1.3 (<0.001) | 37±3.4 (<0.001) | 20±4.0 (<0.001) | 24±5.7 (<0.001) | 0±0.0 (<0.001) |
| <i>Eucalyptus globulus</i> | 67±2.4 (0.998) | 64±1.8 (0.418) | 64±0.8 (0.036) | 61±1.3 (0.037) | 58±5.3 (0.002) | 47±1.5 (<0.001) | 39±6.8 (<0.001) | 40±3.3 (<0.001) |
| <i>Rosmarinus officinalis</i> | 66±1.0 (0.477) | 66±1.4 (0.477) | 62±1.3 (<0.001) | 63±1.9 (<0.001) | 61±1.2 (<0.001) | 26±0.5 (<0.001) | 23±1.3 (<0.001) | 6±4.2 (<0.001) |
| <i>Lavandula angustifolia</i> | 61±1.0 (0.701) | 65±4.0 (0.790) | 59±4.8 (<0.001) | 54±7.0 (0.012) | 34±13.2 (<0.001) | 18±5.7 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Thymus vulgaris</i> | 19±2.4 (<0.001) | 2±3.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Eugenia caryophyllus</i> | 39±15.4 (<0.001) | 15±4.8 (<0.001) | 7±1.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Salvia officinalis</i> | 41±5.6 (<0.001) | 43±5.6 (<0.001) | 25±3.2 (<0.001) | 26±2.8 (<0.001) | 21±1.3 (<0.001) | 15±1.9 (<0.001) | 7.3±0.5 (<0.001) | 0±0.0 (<0.001) |

(a) Results are expressed as mean±SD (n=4).

(b) Data in parentheses are P values of Dunnett test (comparison with control).

volume of 3 $\mu\text{L}/10\text{ mL}$ PDA, while, as expected, the weakest mycelial growth was determined while using 70 $\mu\text{L}/10\text{ mL}$ PDA (Table 2).

The complete inhibition of the mycelial growth was seen in the essential oil of thyme, in the volume of 7 $\mu\text{L}/10\text{ mL}$ PDA, which is followed by the essential oil of clove (9 $\mu\text{L}/10\text{ mL}$ PDA), fennel (15 $\mu\text{L}/10\text{ mL}$ PDA) and peppermint and anise (30 $\mu\text{L}/10\text{ mL}$ PDA). The essential oils of tea and lavender completely inhibited the mycelial growth in the volume of 50 $\mu\text{L}/10\text{ mL}$ PDA, and the essential oils of basil and sage in the volume of 70 $\mu\text{L}/10\text{ mL}$ PDA. Essential oils of rosemary, lemon and eucalyptus did not completely inhibit the mycelial growth even at the highest used volume.

The results show that on the fourth day the essential oils of thyme, clove, fennel, peppermint, anise, tea, true lavender, sage and basil, used in certain volumes, had the same effect as did the fungicide Prosaro 250 EC in the control variable, which completely inhibited the mycelial growth. On the eighth day, just as on the fourth, the measurement results of mycelial growth of *F. oxysporum* showed a decrease in the diameter of the mycelium with the increase of essential oil volume relative to the control variable (Table 3).

Essential oils of thyme, clove, peppermint, tea, sage and basil completely inhibited the mycelial growth in the same

volume as on the fourth day, while essential oils of anise, fennel, and true lavender completely inhibited the mycelial growth of *F. oxysporum* in a greater volume. Essential oils, applied in certain volumes, had the same effect as did the fungicide Prosaro 250 EC in the control variable on the eighth day, which completely inhibited the mycelial growth. Essential oils of rosemary and lemon did not completely inhibit the mycelial growth even in the largest used volume, while the essential oil of eucalyptus did not inhibit the mycelial growth at all.

Measuring the mycelium diameter four days after the inoculation of fungus *B. cinerea*, it was determined that the weakest effect was found with essential oils in the lowest doses, whereas the best effect was when the oils were applied in the volume of 70 $\mu\text{L}/10\text{ mL}$ PDA (Table 4). The complete inhibition of the mycelial growth was seen in the essential oil of thyme in the volume of 9 $\mu\text{L}/10\text{ mL}$ PDA, fennel and peppermint at 50 $\mu\text{L}/10\text{ mL}$ PDA and tea, basil rosemary and sage at 70 $\mu\text{L}/10\text{ mL}$ PDA (Table 4). Those oils did not have the same effect on the mycelial growth in relation to the control as well as the fungicide Switch 62.5 WG.

On the eighth day after the inoculation of fungus *B. cinerea*, the complete inhibition was caused by the essential oil of thyme in the volume of 15 $\mu\text{L}/10\text{ mL}$ PDA, peppermint

Table 3: The relationship between the applied volumes of essential oils ($\mu\text{L}/10\text{ mL}$ PDA) and diameter (mm) of *Fusarium oxysporum* mycelium in which total mycelium inhibition was observed - eight days

| Essential oils | Volumes ($\mu\text{L}/10\text{ mL}$ PDA) and P value (vs Control) | | | | | | | |
|-------------------------------|--|--------------------|--------------------|--------------------|---------------------|---------------------|--------------------|--------------------|
| | 3 | 5 | 7 | 9 | 15 | 30 | 50 | 70 |
| <i>Melaleuca alternifolia</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 72±11.5 (<0.001) | 36±4.1 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Pimpinella anisum</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 47±6.4 (<0.001) | 8±2.6 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Citrus limon</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 76±2.4 (<0.001) | 66±3.9 (<0.001) |
| <i>Mentha x piperita</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 64±4.9 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Foeniculum vulgare</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 35±2.2 (<0.001) | 19±11.3 (<0.001) | 6±4.9 (<0.001) | 0±0.0 (<0.001) |
| <i>Ocimum basilicum</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 78±8.9 (0.003) | 65±5.0 (<0.001) | 55±7.6 (<0.001) | 0±0.0 (<0.001) |
| <i>Eucalyptus globulus</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) |
| <i>Rosmarinus officinalis</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 67±1.7 (<0.001) | 55±2.5 (<0.001) | 31±4.0 (<0.001) |
| <i>Lavandula angustifolia</i> | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 90±0.0 (0.999) | 76±16.0 (0.044) | 65±9.4 (<0.001) | 33±8.7 (<0.001) | 0±0.0 (<0.001) |
| <i>Thymus vulgaris</i> | 42±6.2 (<0.001) | 4±8.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Eugenia caryophyllus</i> | 51±4.9 (<0.001) | 33±7.7 (<0.001) | 11±1.9 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) | 0±0.0 (<0.001) |
| <i>Salvia officinalis</i> | 74±1.3 (<0.001) | 75±2.6 (<0.001) | 47±8.0 (<0.001) | 48±8.1 (<0.001) | 45±2.7 (<0.001) | 34±5.8 (<0.001) | 20±3.5 (<0.001) | 0±0.0 (<0.001) |

(a) Results are expressed as mean±SD (n=4).

(b) Data in parentheses are P values of Dunnett test (comparison with control).

Table 4: The relationship between the applied volumes of essential oils ($\mu\text{L}/10\text{ mL PDA}$) and diameter (mm) of *Botrytis cinerea* mycelium in which total mycelium inhibition was observed - fourth days

| Essential oils | Volumes ($\mu\text{L}/10\text{ mL PDA}$) and P value (vs Control) | | | | | | | |
|-------------------------------|---|--------------------------|--------------------------|---------------------------|---------------------------|---------------------------|--------------------------|--------------------------|
| | 3 | 5 | 7 | 9 | 15 | 30 | 50 | 70 |
| <i>Melaleuca alternifolia</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 21 \pm 5.1 (<0.001) | 16 \pm 3.2 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Pimpinella anisum</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 49 \pm 7.8 (<0.001) | 52 \pm 5.4 (<0.001) | 32 \pm 13.0 (<0.001) | 8 \pm 0.5 (<0.001) | 7 \pm 1.0 (<0.001) | 7 \pm 1.0 (<0.001) |
| <i>Citrus limon</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) |
| <i>Mentha x piperita</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 82 \pm 15.5 (0.921) | 68 \pm 20.5 (0.102) | 50 \pm 27.0 (<0.001) | 7 \pm 1.4 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Foeniculum vulgare</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 55 \pm 19.6 (<0.001) | 59 \pm 9.5 (<0.001) | 8 \pm 1.2 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Ocimum basilicum</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 22 \pm 15.2 (<0.001) | 12 \pm 5.0 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Eucalyptus globulus</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) |
| <i>Rosmarinus officinalis</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 50 \pm 4.9 (<0.001) | 19 \pm 2.9 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Lavandula angustifolia</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 81 \pm 11.8 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 23 \pm 3.1 (<0.001) | 29 \pm 9.0 (<0.001) | 16 \pm 8.3 (<0.001) |
| <i>Thymus vulgaris</i> | 58 \pm 6.4 (<0.001) | 68 \pm 3.3 (<0.001) | 10 \pm 3.6 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Eugenia caryophyllus</i> | 52 \pm 17.4 (<0.001) | 25 \pm 7.2 (<0.001) | 25 \pm 6.5 (<0.001) | 10 \pm 4.0 (<0.001) | 14 \pm 0.5 (<0.001) | 7 \pm 1.3 (<0.001) | 5 \pm 3.5 (<0.001) | 7 \pm 0.6 (<0.001) |
| <i>Salvia officinalis</i> | 90 \pm 0.0 (0.000) | 90 \pm 0.0 (0.999) | 87 \pm 3.8 (0.652) | 68 \pm 2.8 (<0.001) | 54 \pm 7.4 (<0.001) | 21 \pm 2.2 (<0.001) | 17 \pm 2.4 (<0.001) | 0 \pm 0.0 (<0.001) |

(a) Results are expressed as mean \pm SD (n=4).

(b) Data in parentheses are P values of Dunnett test (comparison with control).

Table 5: The relationship between the applied volumes of essential oils ($\mu\text{L}/10\text{ mL PDA}$) and diameter (mm) of *Botrytis cinerea* mycelium in which total mycelium inhibition was observed - eight days

| Essential oils | Volumes ($\mu\text{L}/10\text{ mL PDA}$) and P value (vs Control) | | | | | | | |
|-------------------------------|---|--------------------------|---------------------------|--------------------------|--------------------------|---------------------------|--------------------------|---------------------------|
| | 3 | 5 | 7 | 9 | 15 | 30 | 50 | 70 |
| <i>Melaleuca alternifolia</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 53 \pm 3.4 (<0.001) | 39 \pm 4.9 (<0.001) |
| <i>Pimpinella anisum</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 87 \pm 6.5 (0.745) | 37 \pm 8.1 (<0.001) |
| <i>Citrus limon</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) |
| <i>Mentha x piperita</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 47 \pm 12.8 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Foeniculum vulgare</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 7 \pm 1.0 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Ocimum basilicum</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 59 \pm 9.2 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Eucalyptus globulus</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) |
| <i>Rosmarinus officinalis</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 56 \pm 17.4 (<0.001) |
| <i>Lavandula angustifolia</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 47 \pm 8.7 (<0.001) |
| <i>Thymus vulgaris</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 27 \pm 15.8 (<0.001) | 10 \pm 4.1 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) | 0 \pm 0.0 (<0.001) |
| <i>Eugenia caryophyllus</i> | 90 \pm 0.0 (0.999) | 66 \pm 18.1 (0.006) | 49 \pm 18.7 (<0.001) | 16 \pm 5.1 (<0.001) | 17 \pm 4.7 (<0.001) | 13 \pm 3.1 (<0.001) | 9 \pm 1.6 (<0.001) | 8 \pm 1.4 (<0.001) |
| <i>Salvia officinalis</i> | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 90 \pm 0.0 (0.999) | 65 \pm 6.5 (0.999) | 3 \pm 6.0 (0.999) |

(a) Results are expressed as mean \pm SD (n=4).

(b) Data in parentheses are P values of Dunnett test (comparison with control).

in the volume of 50 µL/10 mL PDA and fennel and basil in the volume of 70 µL/10 mL PDA (Table 5). The oils, both used in those volumes, when compared to the effect of the fungicide Switch 62.5 WG had a better effect on the decrease of the mycelial growth of fungus *B. cinerea*. The essential oils of clove and sage, even though they did not completely inhibit the mycelial growth, had a better effect relative to the control than the fungicide. The essential oils of eucalyptus and lemon did not inhibit the mycelial growth at all, even at highest applied volume.

Estimated values of IC₅₀ parameters for essential oils according to effect on mycelial growth of *Fusarium oxysporum* and *Botrytis cinerea*

Based on the size of diameter of mycelium for every essential oil in all applied volumes, values of IC₅₀ parameters (95 % CI) for *F. oxysporum* and *B. cinerea*.

On the fourth day after the inoculation with fungus *F. oxysporum* the lowest value of IC₅₀ (95% CI) was in the essential oil of thyme 0.36 (0.24 – 0.48), clove 1.03 (0.74 – 1.35), fennel 2.78 (2.28 – 3.36) and anise 3.79 (3.19 – 4.48). The highest IC₅₀ (95% CI) was in the essential oil of eucalyptus 18.7 (14.13 – 24.89) and rosemary 12.42 (10.54 – 14.64) (Figure 1).

On the eighth day after the inoculation with fungus *F. oxysporum* the lowest value IC₅₀ (95% CI) was noted in, as was on the fourth day, in the essential oil of thyme 0.85 (0.54 – 1.21) and clove 1.71 (1.29 – 2.18). They are followed by the essential oils of sage 9.76 (8.27 – 11.52), anise 15.71 (11.57 – 21.39) and fennel 15.84 (11.83 – 21.27). The essential oil of eucalyptus did not inhibit the mycelial growth (Figure 1).

The values of IC₅₀ parameters (95% CI), for the fourth and the eighth day of the experiment, show that in all tested essential oils, the values are larger for *B. cinerea* than for *F. oxysporum*. On the fourth day after inoculation of *B. cinerea*, the lowest IC₅₀ (95% CI) was in essential oil of thyme 2.63 (1.77 – 3.72), clove 2.21 (1.79 – 2.67), anise 9.41 (7.48 – 11.82), peppermint 13.42 (9.82 – 18.37) and fennel 13.82 (10.28 – 18.59). Essential oils of lemon and eucalyptus did not inhibit the mycelial growth (Figure 2).

On the eighth day after inoculation of *B. cinerea*, the lowest IC₅₀ (95% CI) was in the essential oil of thyme 4.93 (3.25 – 7.26) and clove 5.97 (4.51 – 7.83). The essential oil of peppermint had IC₅₀ (95% CI) 25.39 (18.37 – 35.38), and the essential oil of fennel 35.12 (23.78 – 52.87). The largest IC₅₀ (95% CI) was with essential oil of rosemary 151.70 (113.80 – 212.60) and the essential oil of true

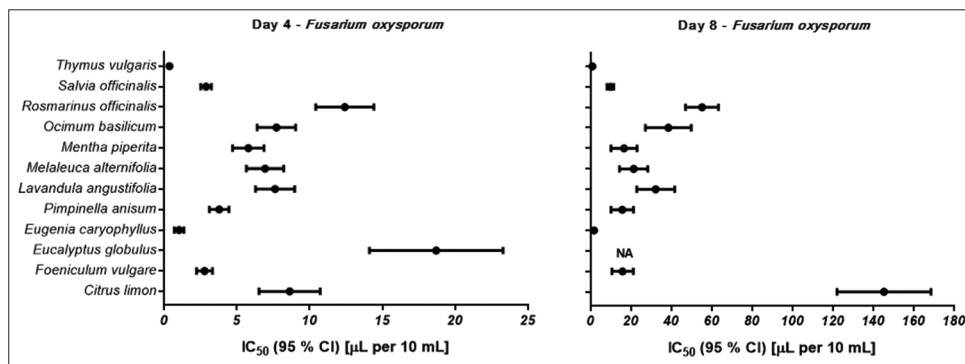


Fig 1. Estimated value of the IC₅₀ parameter for essential oils for fourth and eighth days after inoculation *Fusarium oxysporum*. Data are expressed as the arithmetic mean and 95% confidence interval of the IC₅₀ parameter (n = 32).

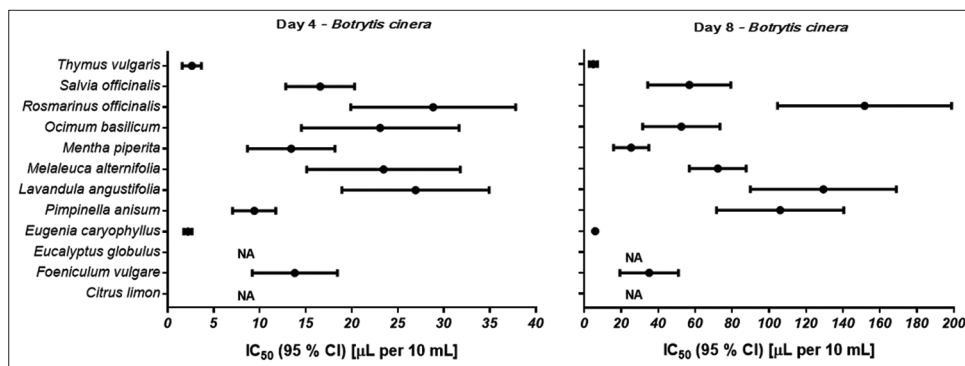


Fig 2. Estimated value of the IC₅₀ parameter for essential oils for fourth and eighth days after inoculation *Botrytis cinerea*. Data are expressed as the arithmetic mean and 95% confidence interval of the IC₅₀ parameter (n = 32).

lavender 129.40 (97.53 – 179.00). Essential oils of lemon and eucalyptus did not inhibit the mycelial growth, just as on the fourth day (Figure 2).

DISCUSSION

Although it was noted that many efforts have been taken and many studies conducted with the aim of achieving a more efficient protection of agricultural crops and adjusting the amount of pesticides used to the real need with a less negative effect on the environment (Villalobos and Fereres, 2016), the possibility of not using plant protection products has never been completely addressed, including the negative effect the products have on the health of people and the pollution of the environment. Using natural plant products, including the essential oils, is a promising alternative aimed at saving the nature, the environment and the human health.

In order to determine the mycelial growth of fungi *Fusarium oxysporum* and *Botrytis cinerea*, as well as the effect of fungicide on pathogens, the relationships between the applied doses of fungicide with control was tested. The results showed a complete growth inhibition of fungus *Fusarium oxysporum* on the fourth and eighth day after the change since changing the Prosaro 250 EC fungicide in all applied doses.

Fungicide Switch 62.5 WG worked in accordance with all the producer's specifications after the fourth day of testing. However, the effect on the *Botrytis cinerea* fungus in *in vitro* conditions kept decreasing and on the eighth day it did not stop it as effectively as we expected.

The results of measuring mycelial growth of the tested pathogens showed a decrease in the growth, depending on the applied volume of essential oils. The achieved results were in the accordance with El-Mohamedy et al. (2013) tests which tested inhibitory activity of some alternatives to fungicides on the growth of eight soil phytopathogenic fungi and determined that the mycelial growth decreases significantly with the increase of applied essential oil concentration. The minimal mycelial growth was determined at the highest concentration, and the complete inhibition was determined at 1.5% concentration of all tested essential oils. Li et al. (2017) determined that the effect of the tea essential oil on the mycelial growth of *B. cinerea* and *Penicillium expansum* depends on the amount of essential oil. At 1.00 mL/L, the essential oil of tea inhibited the growth of *B. cinerea* 77.16% and *P. expansum* 27.77%. The inhibition of the mycelial growth is increased with the increase of the volume of essential oil. With the volume of 1.5 mL of oil/L, the inhibition of *B. cinerea* was 99.02%, while for *P. expansum* the amount of 4.0 mL/L resulted in

98.62% inhibition of mycelial growth. The mycelial growth was completely inhibited with 2.0 and 6.5 mL oil/L for *B. cinerea* odnosno *P. expansum*. The achieved results of our research is also in accordance with results of many other scientists who tested the antifungal activity of essential oils on phytopathogenic fungi and who claim that their activity depended on the applied volume, that is, concentration (Palfi et al., 2018; Duduk at al., 2015; Adabayo et al., 2013; Hung et al., 2013; Lu et al., 2013; Moghaddam et al., 2013; Nguyen et al., 2017; Viuda-Martos et al., 2008). However, antifungal properties of essential oils depend on the properties of plants they are extracted from (Piyo et al., 2009) and their chemical composition (Ćosić et al., 2014).

The positive effect of essential oil of thyme in this research is in a accordance with the research of Alam et al. (2014) who claim that the essential of *Thymus capitatus* L. in the volume of 2 µg/mL completely inhibited the growth of *Fusarium oxysporum*, *Alternaria solani*, *Aspergillus niger*, *Penicillium sp1* and *Penicillium sp2*. Marandi et al. (2011) determined that *Thymus kotschyannus* had the strongest antifungal activity in *in vitro* conditions and completely inhibited the fungal growth in volumes above 200 µL/L. Furthermore, Elshafie et al. (2015) tested potential fungistatic or fungicidal activity of essential oil of thyme (*Thymus vulgaris*) and verbena (*Verbena officinalis*) in *in vivo* conditions in various concentrations against *Monilinia laxa*, *Monilinia fructigena* and *Monilinia fructicola*. They determined that the greatest applied volumes of the essential oil of verbena (1000 ppm) and thyme (500 ppm) considerably reduced the diameter of brown lesions while smaller volumes of the essential of verbena (500 ppm) and thyme (250 ppm) obtained low effect. A good antifungal activity of essential oils of thyme and anise on the increase of mycelial growth of twelve phytopathogenic fungi was also determined by Ćosić et al. (2010).

In our research, the essential oils of lemon and eucalyptus did not inhibit the mycelial growth of *B. cinerea* at all. Moreover, those oils did not completely inhibit the mycelial growth of *F. oxysporum* even at highest applied volumes. Our results are not in accordance with the research of Viuda-Martos at al. (2008) who claim that the essential oil of citrus show a certain antifungal activity, and the research of Lee at al. (2007) who claim that the eucalyptus oil (*Eucalyptus citriodora*) has a strong antifungal effect on the mycelial growth of fungus *B. cinerea* and that the inhibition grows with the increase of the applied volume of the essential oil.

In our research, the essential oils of thyme, clove, fennel, peppermint, anise, tea, true lavender, sage and basil were applied in volumes which completely inhibited the mycelial growth of *F. oxysporum*, and had the same effect relative to

the control as the fungicide Prosaro 250 EC. The complete inhibition of mycelial growth of *B. cinerea* at the end of the experiment caused by essential oils of thyme, fennel, peppermint and basil. Those oils had a better effect on the mycelial growth relative to control than the fungicide Switch 62.5 WG. The essential oils of clove and sage, although they did not inhibit the mycelial growth, had a better effect relative to control than a fungicide.

Therefore, after comparing the effect of essential oils with the effect of chemical fungicides, it can be concluded that in certain volumes essential oils could have the same or better inhibitory effect on the mycelial growth than fungicides, which is in accordance with the results of Moghtader et al. (2011) and Sitara et al. (2008).

The decreased antifungal activity of essential oils in this paper, which was determined in the last measurement, is in accordance with results of Nosrati et al. (2011), who state that the inhibitory effect depends on the volume of essential oils, but also the duration of inhibition. The author states that the samples were treated with 1 μ L of essential oil of mint showed a slow decrease on the inhibition of mycelial growth of *Fusarium oxysporum* f. sp. *radicis-cucumerinum* throughout the incubation period. Similar results were stated by Sumalan et al. (2013) and Adabayon et al. (2013).

IC₅₀ (95% CI) parameter values were calculated based on the diameter of mycelium for each essential oil in all applied volumes for *Fusarium oxysporum* and *Botrytis cinerea*.

On the fourth day of the experiment, it was determined that the lowest IC₅₀ (95% CI) for *Fusarium oxysporum* was in the essential oils of thyme, clove, fennel and anise, and the highest values of the essential oils of eucalyptus and rosemary. On the eighth day, the lowest IC₅₀ (95% CI) was in the essential oils of thyme, clove, sage, anise and fennel. The essential oil of lemon had the highest value of IC₅₀ (95% CI), while the essential oil of eucalyptus did not inhibit the growth of *F. oxysporum*. On the fourth day after the inoculation of mycelium of *Botrytis cinerea* the lowest IC₅₀ was in the essential oils of thyme, clove, anise, peppermint and fennel. On the eighth day, the lowest IC₅₀ was in the essential oils of thyme, clove, peppermint and fennel, while the essential oils of lemon and eucalyptus did not inhibit the mycelial growth at all, not even in the highest applied volumes. Also, an increase of IC₅₀ was noted on the eighth day in relation to the fourth day in both tested phytopathogenic fungi.

Bi et al. (2012) stated that among the fourteen tested commercial essential oils, the essential oils *Origanum syriacum*, *Cymbopogon martini* and *Thymus vulgaris* had the lowest values of

EC₅₀ (< 0.15 μ g/mL). Perez-Sanchez et al. (2007) determined by testing antifungal properties of essential oils in six *Thymus zygis* Loeff. ex L. on phytopathogenic fungi *Phytophthora irregularis*, *Rhizoctonia solani*, *Colletotrichum acutatum*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum* that the EC₅₀ is in the range between 86 ppm and 577 ppm. Good antifungal activity of essential oil *Thymus vulgaris* on *B. cinerea* in the amount of 500 μ L/mL was also determined by Gebel and Magurno (2014).

Huang et al. (2010) states that during the testing of antifungal activity of essential oil of star anise (*Illicium verum*) and its main component trans-anethole, the IC₅₀ for all isolates of *F. oxysporum* was between 0.14 and 0.20 mg/mL for trans-anethole, and for the oil it was 0.16, to 0.25 mg/mL.

The results of our research have showed that the essential oil of eucalyptus has the highest IC₅₀ which is in accordance with the results of Sharma et al. (2017) that state that the eucalyptus oil shows inhibitory activity at relatively high concentrations and that IC₅₀ for the essential oil of eucalyptus was 207.86 ppm. On the other hand, Derwich et al. (2013) state that the essential oil *Eucalyptus globulus* has a good antifungal effect on the fungus *Penicillium citrinum* and that the minimum inhibitory concentration was 3.07 and 96.14 μ L/mL.

Fonseca et al. (2015) state that the essential oils *Mentha piperita* and *Rosmarinus officinalis* had MIC₅₀ 0.44 mg/mL, and MIC₉₀ 3.5 mg/mL for *Pythium insidiosum*. Moghaddam et al. (2013) determined that the essential oil *M. piperita* in *in vitro* conditions and in the volume of 800 ppm and 1600 ppm completely inhibited the mycelial growth of *Dreschlera spicifera*, and *Fusarium oxysporum* f.sp. *ciceris* in the concentration of ppm, while Sharma et al. (2017) state the IC₅₀ values for essential oil of mint from 60.05 ppm. Authors state that the essential oils of mint show inhibitory activity at relatively higher concentrations.

Comparing the estimated values of IC₅₀ (95% CI) parameters of essential oils according to their effect on *F. oxysporum* and *B. cinerea* through the entire duration of the experiment, it is visible that with all the tested essential oils, the IC₅₀ (95% CI) values are higher for *B. cinerea* than for *F. oxysporum*. The results of this research are in accordance with research done by authors who claim that essential oils have different effects, depending on the type of phytopathogenic fungus (Karimi et al., 2016; Türkölmez and Soyulu, 2014; Moghaddam et al., 2013; Amini et al., 2012; Bahraminejad et al., 2011; Ćosić et al., 2010) and the incubation time (Al-Reza et al., 2010). Li et al. (2017) state that in *in vitro* conditions, fungus *B. cinerea* is more sensitive to essential oil *Melaleuca alternifolia* than *Penicillium expansum*. Also, a higher sensitivity of *B. cinerea* was also

determined in *in vivo* conditions with artificial infection. Various antifungal activity to certain fungi was also noted by Angioni et al. (2006) who tested the activity of essential oil *Lavandula stoechas* L. ssp. *stoechas* and its components. They determined a strong antifungal activity of the essential oil on pathogenic fungi *Rhizoctonia solani* and *Fusarium oxysporum*, and less effective on *Aspergillus flavus*.

The results of the research with the same essential oils, plant types and pathogen microorganisms can be very different (Hyldgaard et al., 2012) and it is necessary to conduct continuous research, especially in combination with other antimicrobial compounds which enable synergistic effect (Adeyinka and Richard, 2015) or innovative methods of application.

CONCLUSIONS

Based on the conducted *in vitro* research of antifungal activity of twelve essential oils on the mycelial growth of *Fusarium oxysporum* and *Botrytis cinerea* in different volumes and achieved results, we can conclude that some essential oils had the same negative effect on the mycelial growth relative to the control as fungicide Prosaro 250 EC and Switch 62.5 WG. The antifungal effect of essential oils is decreased with the duration of the incubation, while with the increase of applied volumes of essential oils, the mycelial growth of *F. oxysporum* and *B. cinerea* is slowed down. The best antifungal effect on the tested phytopathogenic fungi was the essential oil of thyme. That essential oil completely inhibited the mycelial growth of both phytopathogenic fungi in the smallest applied volume on the eighth day of inoculation.

Authors' Contributions

Marina Palfi, PhD conducted a laboratory research and processed and interpreted the results. Statistical data processing was done by Pasko Konjevoda, PhD. Prof. Dr. Karolina Vrandečić worked on laboratory research and interpretation of results. Prof. Dr. Jasenka Čosić designed the research (material and methods) and compared our results with the results of other authors.

We believe that the results of this research will be the basis for further research on the conditions *in vivo* and the possible development of new biofungicides.

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