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ALTERNATIVE APPROACH IN CONTROL OF TOMATO PATHOGEN BY USING ESSENTIAL OILS *IN VITRO*

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Abstract

Essential oils of *Mentha piperita, Eucaliptus globulus, Pinus sylvestris, Rosmarinus officinalis, Pimpinella anisum* and *Origanum vulgare* were studied for antifungal effect of their volatile phase toward tomato pathogen (*Fusarium oxysporum* f.sp. *lycopersici*) *In vitro*. Antifungal effect of oils was expressed through calculating percentage of inhibition of radial growth of mycelia of pathogen, and by determining MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration). Results indicated that all of examined oils expressed antifungal activity in different concentrations. Essential oil of oregano (*Origanum vulgare*) was the most efficient in inhibition of mycelial growth with total inhibition applied at lowest concentration of 0.04 µl/ml of air. MIC and MFC for this oil were also found at 0.04 µl/ml of air. Oils of anise (*Pimpinella anisum*) and menthe (*Mentha piperita*) were also very effective. Anise oil had totally inhibited mycelium applied at 0.1 µl/ml of air. MIC value was 0.3 µl/ml of air, while MFC was higher than maximal applied concentration (>0.6 µl/ml of air). Menthe oil had total inhibition of mycelial growth applied at 0.3 µl/ml of air and the MIC value was at the same point. MFC was higher than maximal applied concentration (>0.6 µl/ml of air). Other essential oils expressed high inhibition rate but it wasn't 100%. The results of this research indicate that essential oil of oregano followed by oils of menthe and anise has potential to suppress the mycelial growth *Fusarium oxysporum* f. sp. *lycopersici*, *In vitro*. Further research should provide answers for final goal – applying essential oils *In vivo*.

Introduction

Causer of Fusarium wilt of tomato (FWT) *Fusarium oxysporum* Schlechtend: Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen is, economically, one of the most important and widespread diseases of the cultivated tomato (*Solanum lycopersicon*) (Reis *et al*., 2005). It is a soilborne pathogen in the class Hyphomycetes that causes FWT as the only host of pathogen (Rai *et al*., 2011). This pathogen have three races 1, 2 and 3 and have a longterm persistance via three types of asexual spores *viz.,* microconidia, macroconidia and thick wall chlamidospores. Usually, once infested soil remains so indefinitelly (Agrios, 2005). The thick walls of chlamidospores, together with their storage reserves, make them the most persistent spore form which can survive several years in the soil until the presence of healthy plants is detected (Beckman, 1987). After detecting host presence germination of spores is stimulated and germ tubes from spores or mycelia penetrate root tips either directly or through wounds. Mycelium enters the xylem vessels branches and produces microconidia, which are carried upward in the sap stream. Vessel clogging by mycelium, spores, gels, gums and tyloses as well as the crushing of vessels by proliferating adjacent parenchyma cells eventually cause death of tomato plant (Agrios, 2005).

This pathogen was controlled in the past mainly through the fumigation of soil with methyl bromide until it was found to be environmentally damaging and because of that methyl bromide was banned for use by Montreal Protocol (Lim *et al*., 2006). Taking into consideration the need to preserve ecosystems and a growing awareness of the possible harmful effects of chemicals, it was necessary to find a long-term solution without the harmful impact on the ecosystem, on one hand, and to protect this important production, on the other (Đorđević *et al.,* 2012). One of the possible solutions for control is the appliance of essential oils of some aromatic and medicinal plants. Since the plants are the source of large number of substances with fungicide and fungstatic effect, they could be the mentioned solution (Ajaib *et al.,* 2011; Aslam *et al*., 2010; Isman, 2000; Naz *et al.,* 2010; Sitara *et al.,* 2011; Tanović *et al*., 2005; Ting-Ting *et al.,* 2011). Many researches examined the influence of essential oils on fungi, pathogens of plants, and fungi important for food industry, proved that these plant compounds could be the solution (Parveen *et al*., 2010; Sitara *et al*., 2008; Sitara *et al.,* 2011; Mehjabeen *et al.,* 2011; Soković *et al*., 2009; Tanović *et al*., 2009; Veljić *et al*., 2009). Before any solution for pathogen control could be applied *in vivo*, biological or conventional, it is first necessary to determine its toxicity, i.e. its efficiency *In vitro*. Therefore, the present study was mainly aimed to determine the efficacy of essential oils of *Mentha piperita*, *Eucaliptus globulus*, *Pinus sylvestris*, *Rosmarinus officinalis*, *Pimpinella anisum* and *Origanum vulgare* in control of *Fusarium oxysporum* f. sp. *lycopersici*, *In vitro*.

Materials and Methods

Pathogen: Fungus was isolated from tomato plants with typical fusarium wilt symptoms. Samples of lower stem of tomato plants were first rinsed in the flow of running tap water. After drying on sterile filter paper samples were surface disinfested with 96% alcohol for a few seconds and washed with sterile-distilled water and dried in the sterile flow of air in flow hood. Isolations were performed by placing plant tissues from the transition area between necrotic and non-necrotic area of xylem, on the PDA and incubated for 7 days until pathogens mycelia appear. After forming fungal colony from single-spore, isolates were kept on the PDA in the refrigerator on 4°C until further use. After examination under light microscope isolates were identified as *Fusarium oxysporum* and after testing pathogenicity by inoculation and re-isolation, isolates were identified as tomato pathogen *viz., Fusarium oxysporum* f. sp. *lycopersici*.

Antifungal effect of essential oils: Six plant essential oils ***viz.,*** *Mentha piperita*, *Eucaliptus globulus*, *Pinus sylvestris*, *Rosmarinus officinalis*, *Pimpinella anisum* and *Origanum vulgare* were used in the present study. The effect of these oils toward FWT has been determined by exposure of pathogen to volatile phase of oils (Tanović *et al*., 2009; Soylu *et al*., 2006). Mycelia plug of the pathogen (5x5mm) was transmitted to the center of petri plate (R=9 cm), after which the plate has been turned upside down. Oils were inflicted on a sterile paper disk (R=0.5 cm), located at the centre of petri plates. Various concentrations of oils ***viz.,*** 0.04, 0.06, 0.1, 0.15, 0.3 and 0.6 µl/ml of air were tested in the petri plates. In order to facilitate the contact of volatile phase of oils and pathogens, petri plates were kept upside down. The plates were covered with self-adhesive foil to prevent release of oil vapors out of the plates. Petri plates were kept at 24°C. Petri plates with a drop of sterile distilled water instead of oil were used as control. After four days, the growth of colonies has been measured and on the basis of these values the percentage of inhibition of colony growth has been calculated. Seven days after this trail the inhibitory effect of oil on the pathogen has been determined. The concentration of oil that completely inhibited the growth of the pathogen mycelium was considered fungistatic and the lowest such value was determined as the minimum inhibitory concentration (MIC). The petri plates in which an inhibitory effect was found, were ventilated in a sterile laminar flow of air over a period of 30 minutes in order to completely remove a volatile oil phase, and then the petri plates were kept for seven days in a thermostat on the same temperature as previously. This is done to determine the lethal concentration of oil. The concentration of oil that suppresses mycelium growth even after 7 days after ventilation was taken as lethal. The lowest such concentration was the minimum fungicidal concentration (MFC).

Statistical analysis: All trials have been set twice with five replications of each oil concentration. Percentage of inhibition of mycelia growth were calculated using following formula:-

$$(\%) \quad = \quad (g_c - g_t / g_c) \quad x \quad 100$$

where, *gc* is the growth of mycelium in control plates, *gt* is the growth of mycelium in treated plates.

Results

Antifungal activity of essential oils: The obtained results showed that oils had different level of inhibition on mycelia growth of FWT in different concentrations. Essential oils of oregano (Origanum vulgare), anise (*Pimpinella anisum*) and menthe (*Mentha piperita*) had strong inhibition level followed by oils of eucalyptus (*Eucaliptus globulus*) and rosemary (*Rosmarinus officinalis*). Meanwhile essential oil of *Pinus sylvestris* had weak inhibition level. Essential oils of oregano expressed 100% inhibition of FWT in all of the tested concentrations (Fig. 1). Oils of menthe and anise had inhibited mycelial growth of FWT but in higher concentrations. Radial growth of FWT was totally inhibited by anise at 0.1 µl/ml of air, meanwhile menthe oil had total inhibition of mycelium growth applied at 0.3 µl/ml of air.

Toxicity of essential oils: Measurements of growth of pathogens mycelia after 7 days of treatment showed that oils had different degree of toxicity to this pathogen (Table 1). Seven days after exposure of pathogen to essential oils minimum inhibitory concentration (MIC) was determined. Essential oil of oregano had MIC applied at 0.04 µl/ml of air (Fig. 2). Meanwhile, oils of anise and menthe expressed MIC applied at 0.3 µl/ml of air. Essential oils of eucalyptus, rosemary and pine had MIC value greater than highest applied concentration (>0.6 µl/ml of air). Oils that expressed total inhibition of mycelial growth of pathogen, after 7 days from exposure, were air-removed and plates were kept for seven more days to determine the minimum fungicidal concentration (MFC). It appears that only essential oil of oregano expressed high toxicity and total inhibition of radial growth of mycelia even without presence of essential oil after that period of time (Table 1).

Discussion

The increasing social and economic implications caused by fungi means there is a constant striving to produce safer food and to develop new antifungal agents (Feng & Zheng, 2007). Antimicrobial activity of essential oils of aromatic and medicinal plants has been recognized for long time. Strong antimicrobial effect of some essential oils has been previously reported (Đorđević ***et al.,*** 2011; Feng & Zheng, 2007; Lee *et al*., 2007; Soylu *et al*., 2006; Tanović *et al*., 2005, 2009; Veljić *et al*., 2009; Džamić *et al*., 2008a&b). Comparison of results of different studies is difficult because of differences in plant extract composition and in methodologies of assessments of microbial activity (Arslan & Dervis, 2010).

Results of the present study showed that all of the tested oils can suppress growth of mycelia of *Fusarium oxysporum* f. sp. *lycopersici*. Essential oils of oregano and anise expressed the highest antifungal activity against FWT. Volatile compounds of oregano essential oil has been reported as strong inhibitor of mycelia growth of some soil-borne pathogens *Fusarium solani* var. *coeruleum*, *Fusarium oxysporum*, *Pythium ultimum*, *Rhizoctonia solani*, *Verticillium dahliae* as well as *Botrytis cinerea* and *Phytophora infestans*, where pathogens were totally inhibited at minimum concentrations (Arslan & Dervis, 2010; Daferera *et al*., 2003; Lee *et al*., 2007; Soylu *et al*., 2006). Volatile phase of essential oils of anise was reported to be highly effective in inhibition of mycelial growth of *Botrytis cinerea* having fungicidal effect applied at 0,08 µl/ml of air, and mushroom pathogen *Mycogone perniciosa* having fungicidal effect applied at 0.04 µl/ml of air (Tanović *et al*., 2005, 2009). Some other studies indicated effectiveness of these essential oils to food-borne diseases as well (López *et al*., 2007; Suhr & Nielsen, 2003).

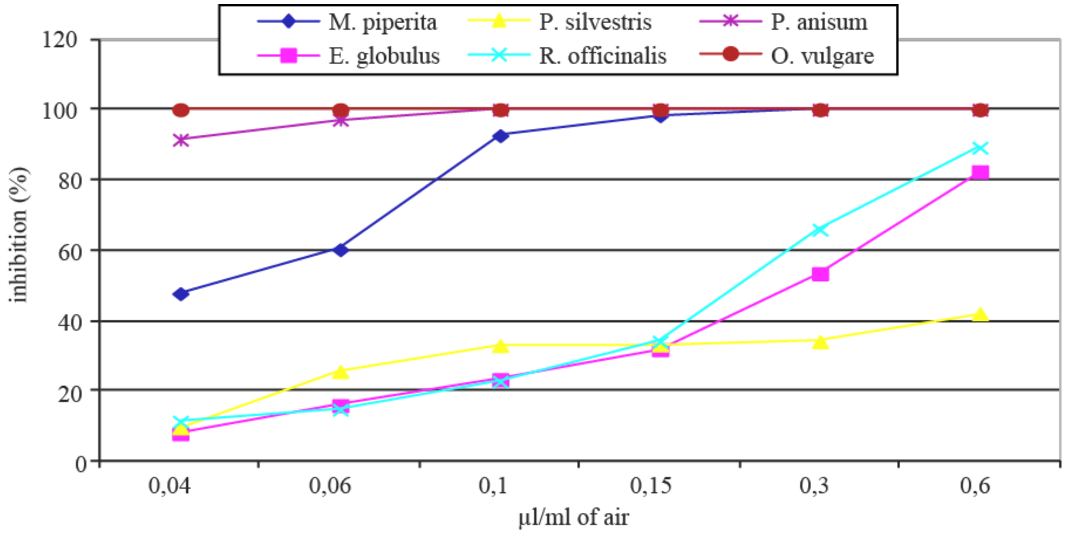

Fig. 1. Effectiveness of essential oils in inhibition of radial mycelia growth of FWT after four days of exposure.

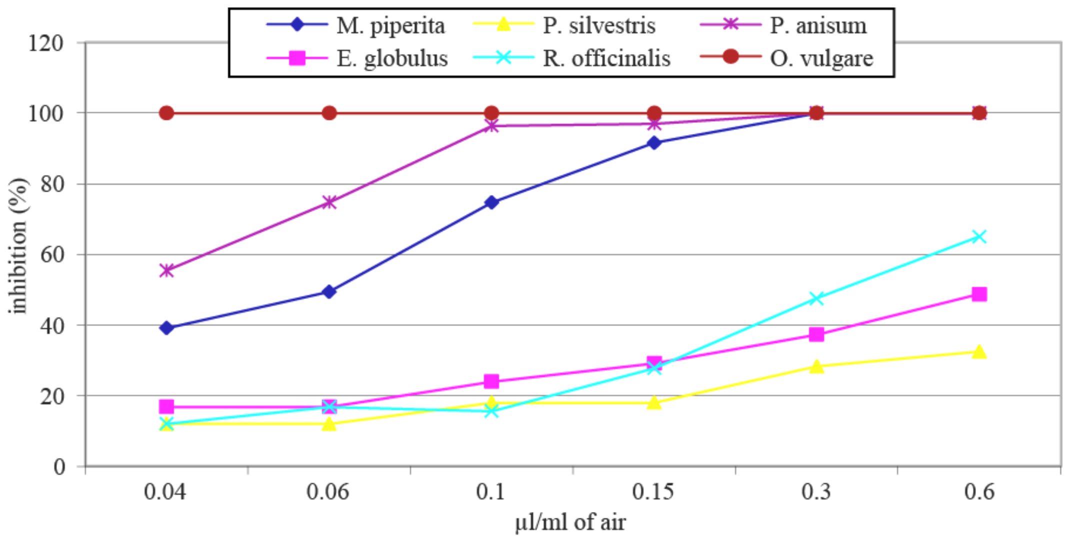

Fig. 2. Effect of essential oils on radial growth of *Fusarium oxysporum* f.sp. *lycopersici* after seven days exposure.

Table 1. Toxicity of essential oils to *Fusarium oxysporum* f. sp. *Lycopersici*.

Essential oils	Minimal inhibitory concentration (MIC[x])	Minimal fungicidal concentration (MFC[y])
Mentha piperita	0,3 µl/ml of air	> 0,6 µl/ml of air
Eucaliptus globules	> 0,6 µl/ml of air	> 0,6 µl/ml of air
Pinus sylvestris	> 0,6 µl/ml of air	> 0,6 µl/ml of air
Rosmarinus officinalis	> 0,6 µl/ml of air	> 0,6 µl/ml of air
Pimpinella anisum	0,3 µl/ml of air	> 0,6 µl/ml of air
Origanum vulgare	< 0,04 µl/ml of air	< 0,04 µl/ml of air

[x] Minimal concentration of oil that cause complete inhibition of mycelia growth after seven day exposure (minimum inhibitory concentration)
[y] Minimal concentration of oil that cause lethal effect on pathogen (minimal fungicidal concentration)

Mode of action of these essential oils was subject of numeral studies but it is still not completely understood (Chang *et al*., 2001; Ultee *et al*., 2002). Due to the fact that the main compounds of the oregano essential oil are carvacrol, thymol, c-terpinene and p-cymene (Daferera *et al*., 2003; Soylu *et al*., 2006). Thus we can assume that these compounds are the main holders of antimicrobial activity and carriers of mode of action of this oil. In order to confirm this assumption more detail research based on the effect of these compounds alone on the observed pathogen must be performed. Those results will give more precise facts regarding their antimicrobial effect.

The results of this research indicate that essential oil of oregano but also oils of menthe and anise have potential to suppress *Fusarium oxysporum* f. sp. *lycopersici*, *In vitro*. Therefore these oils could be used for control of these pathogens *in vivo* and the plant rich with these oils could be an important part of biocontrol strategy. Influence on these pathogens *in vivo* and on tomato plants as well, will be a subject of future research.

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