IN VITRO ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS AND HOMEMADE FUNGICIDES ON GROWTH OF *PHYTOPHTHORA PARASITICA* DASTUR.

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Abstract. Several essential oils (EOs) and homemade fungicides were evaluated for their efficacy as potential fungicides against *Phytophthora parasitica*. Not all EOs were found to kill or inhibit the growth of *P. parasitica* mycelium, but when found, it was on a dose-depended manner. The aim of this paper was to find substitutes to synthetic fungicides currently used in the control of oomycete pathogen *P. parasitica*. The antifungal activity of 22 commercially purchased essential oils, along with homemade fungicides based on baking soda, garlic and hydrogen perodixe were investigated against *P. parasitica*. For the homemade fungicides, we have selected the most used recipes by international farmers. EOs are complex mixtures and we observed a synergistic activity ascriptable to the phytocomplex, but also the activity of some major components. This may lead to the differences observed in our tests. The common compounds of EOs that had an influence over the growth of *P. parasitica* mycelium are eugenol, eugenol acetate, iso-eugenol, borneol, dipetene, limonene and β-caryophyllene. As the present paper showed, the oil concentrations necessary to kill *P. parasitica* are most of the times much higher than those required to inhibit their growth.

Keywords: *Phytophthora parasitica*, essential oil, inhibition, mycelial growth, antifungal activity

Abbreviations
EO(s) – essential oil(s)
B1 – bicarbonate recipe no. 1
B2 – bicarbonate recipe no. 2
Us (Ga) – garlic recipe
AO(HP) – hydrogen peroxide recipe

INTRODUCTION

Oomycetes are morphologically similar to true fungi, but from a phylogenetical stand point, they are distant (Meng et al., 2014). Over 100 species are included in the genus *Phytophthora* (Kroon et al., 2012), and the number is still increasing.

Oomycetes distinguish themselves from true fungi (Latijnhouwers et al., 2003) through a series of characteristics, such as oomycete hyphae are multinucleated, nonseptate while fungi hyphae are septate and oomycetes are diploid while fungi are haploid. There are noticeable differences in cell wall composition also: oomycetes' consist primarily of 1,3-b-glucans, few 1,6-b-glucans and 1,4-b-glucans while fungi cell wall structure is made of chitin (Werner et al., 2002). Fungicides usually target the synthesis of chitin and sterol, meaning they are ineffective in controlling oomycete diseases.

Phytophthora parasitica Dastur (syn. *Phytophthora nicotianae* Breda de Haan) is a typical root pathogen, being capable of infecting a broad host range of over 72 plant genera (Hickman, 1958), so it is essential to develop disease-control measures. The development of resistance to the already reduced number of fungicides (Holmes and Eckert, 1999), along

with the concerns over pollution and the needs of organic farmers to protect their crops led us to explore new alternatives among essential oils (EOs) of aromatic plants. In the last years an increasing interest in the antifungal properties of EOs as substitutes for conventional synthetic pesticides has been registered (Tabassum and Vidyasagar, 2013). EOs have been long recognized for their antiviral, antibacterial, antifungal, insecticidal and antioxidant properties and lately they drive the research community to find new uses and applications of these substances. Investigations show promising activities against many pathogens when tested in vitro.

EOs or aromatic plant essences are volatile and fragrant substances produced by plants, with an oily or lipid-like consistency (Pichersky et al., 2006). The majority of them are liquid at room temperature, but some are solid or resinous, and show colors ranging from pale yellow to emerald green, from blue to dark brownish red (Balz, 1999). They are synthesized by all plant organs, i.e., buds, flowers, seeds, fruits, leaves, stems, roots, bark or wood and are stored in secretory cells, cavities, canals or epidermic cells (Bakkali et al., 2008).

EOs are not simple compounds or only simple mixtures of several individual compounds. They may contain up to 100 components, but most of them contain about 20 to 60 (Pengelly, 2004; Langenheim, 1994; İşcan et al., 2005; Dung et al., 2008).

Several EOs and homemade fungicides were evaluated for their efficacy as potential fungicides against *Phytophthora parasitica*. Agar-dilution method was used in which 1 mL of serial dilution was inoculated at concentrations of 0.1%, 1% and 10%. A 7 mm mycelial plug was centered in each 85 mm petri dish and after 24h the mycelium size was measured daily for a period of 36 days until no growth was registered. Not all EOs were found to kill or inhibit the growth of *P. parasitica* mycelium, but when found, it was on a dose-depended manner.

The aim of this paper was to find substitutes to synthetic fungicides currently used in the control of oomycete pathogen Phytophthora parasitica, the causal agent of tomato root rot. In view of these considerations, the present work investigated the antifungal activity of 22 EOs and a few homemade fungicides, underlining those which showed the best antifungal profile, information which will be used later for an in vivo study against *P. parasitica* on greenhouse tomatoes.

MATERIALS AND METHODS

The antifungal activity of 22 commercially purchased essential oils, i.e. anise (*Pimpinella anisum* L.), basil (*Ocimum basalicum* L.), Indian frankincense (*Boswellia serrata* T.), cinnamon (*Cinnamomum aromaticum* L.), camphor tree (*Cinnamomum camphora* L.), lemongrass (*Cymbopogon winterianus* L.), cloves (*Syzygium aromaticum* L.), coriander (*Coriandrum sativum* L.), May Chang (*Litsea cubeba*), fennel (*Foeniculum vulgare* M.), oil grass (*Cymbopogon citratus* DC.), lavender (*Lavandula angustifolia* Mill.), tea tree (*Melaleuca viridiflora* Sol.), orange (*Citrus* x *sinensis* L.), palmarosa (*Cymbopogon martinii* Roxb.), turmeric (*Curcuma longa*), rosemary (*Rosmarinus officinalis* L.), clary sage (*Salvia sclarea* L.), spearmint (*Mentha spicata* L.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.) and lemon (*Citrus limon* L.), along with homemade fungicides based on baking soda, garlic and hydrogen perodixe were investigated against *P. parasitica*.

For the homemade fungicides, we have selected the most used recipes by international farmers: mix 5 tablespoons of baking soda with 1 teaspoon of liquid soap in 1 gallon of water (B1); mix 1 tablespoon of baking soda with 1 teaspoon of castor oil in 1

gallon of water (B2); chop 100 g of garlic cloves in 1 L of water Us(Ga); hydrogen peroxide 3% AO(HP).

For the experiment, 1 mL of EO dilution or homemade fungicide was pipetted in each Petri dish (85mm), after which 17 mL of potato dextrose agar (PDA, Scharlau, Spain) was added at temperature of 50ºC, to avoid volatilizing or denaturing the aromatic compounds in the oils, then the dishes were stirred for 20s. EO concentrations of 0.1%, 1% and 10% were expressed using Percent Composition by Mass (%), in which the mass of the solute is divided by the mass of the solution (mass of solute plus mass of solvent), then multiplied by 100. Media was allowed to cool and solidify. After 2h, a 7 mm mycelial plug of *P. parasitica* (Strain number MUCL 41915, BCCM, Belgium) was centered onto each Petri dish. The *P. parasitica* cores were taken from the edge of individual 14 days old colonies. All Petri dishes were left inside the laminar-flow hood for 24h then stored inverted so that water would not condense on the agar surface. Dishes were incubated in the dark inside a 27ºC germination chamber. A total of 66 dishes were used per replication, with 3 replications. Control plates were included in each replication: 3 PDA media plates inoculated with the pathogen, to determine the viability and growth.

Fungal growth measurements were taken every 24h for 36 days, until no fungal growth was registered. Two Way ANOVA was used to determine the effect of treatments on each concentration on growth measurements. Statistical analysis was performed with the IBM SPSS Amos v20.

RESULTS AND DISCUSSIONS

EOs are complex mixtures and we observed a synergistic activity ascriptable to the phytocomplex, but also the activity of some major components. This may lead to the differences observed in our tests.

Fig. 1 shows the growth of mycelium size in mm by the end of the 36 day recording period. The most satisfying result of EO activity was obtained when using cinnamon EO (*Cinnamomum aromaticum* L.): at the concentration of 0.1% the mycelium grew slowly to 18 mm in diameter; at 1% concentration mycelium diameter was of 14 mm while at 10% the growth was of maximum 11 mm. The main components of *C. aromaticum* EO are eugenol, eugenol acetate, cinnamic aldehyde and benzyl benzoate. The hydrogen peroxide 3% AO(HP) killed the fungus.

Lemongrass (*Cymbopogon winterianus* L.), coriander (*Coriandrum sativum* L.), palmarosa (*Cymbopogon martinii* Roxb.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.), cloves (*Syzygium aromaticum* L.) EOs demonstrated cidal activity against *P. parasitica* at the highest concentration. Among the common compononets, we found eugenol and eugenol acetate in *C. aromaticum* and *S. aromaticum*, which also contains iso-eugenol. *C. winterianus* and *C. sativum* have borneol and dipetene as correspondent components. Limonene is an element found in *C. winterianus* and *C. martinii* which incorporates dipetene also. β-caryophyllene is found both in *T. vulgaris* along with borneol and in *O. vulgare*.

Oil grass (*Cymbopogon citratus* DC) and rosemary (*Rosmarinus officinalis* L.) EOs share limonene and both showed a medium inhibitory reaction. May Chang (*Litsea cubeba*), turmeric (*Curcuma longa*) and baking soda recipe no. 1 (B1) treatments showed, as well, an average growth of *P. parasitica*.

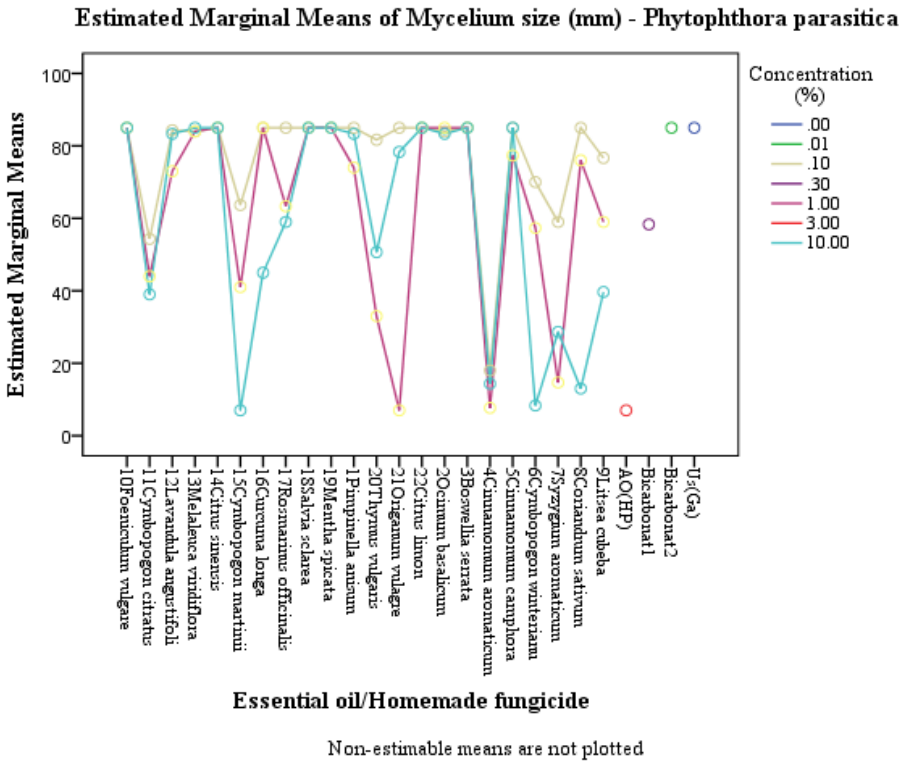

Fig. 1. Effect of EOs and homemade fungicides on *Phytophthora parasitica* mycelium size at different concentrations (original)

All the others EOs, i.e. anise (*Pimpinella anisum* L.), basil (*Ocimum basalicum* L.), Indian frankincense (*Boswellia serrata* T.), camphor tree (*Cinnamomum camphora* L.), fennel (*Foeniculum vulgare* M.), lavender (*Lavandula angustifolia* Mill.), tea tree (*Melaleuca viridiflora* Sol.), orange (*Citrus* x *sinensis* L.), clary sage (*Salvia sclarea* L.), spearmint (*Mentha spicata* L.), lemon (*Citrus limon* L.), baking soda recipe no. 2 (B2) and garlic homemade fungicide [Us(Ga)] did not affect the growth of *P. parasitica* mycelium.

From a time-series point of view, the evolution of *P. parasitica* mycelium was distinct under treatment with lemongrass (*Cymbopogon winterianus* L.) (Fig. 2.) and oil grass (*Cymbopogon citratus* DC) (Fig. 3.).

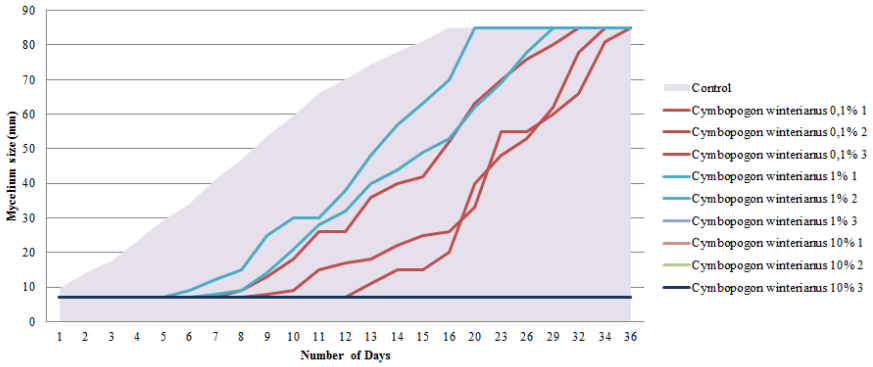

Fig. 2. Effect of *Cymbopogon winterianus* EO on *Phytophthora parasitica* mycelium size at different concentrations (original)

At 0.1% concentration, *P. parasitica* did not show any growth for 7 to 11 days, after which it started to grow until 85 mm in diameter. The fungus was inhibited for 5 – 6 days at the following testing concentration, then it reached the maximum size. At 10% oil concentration, the fungus was killed.

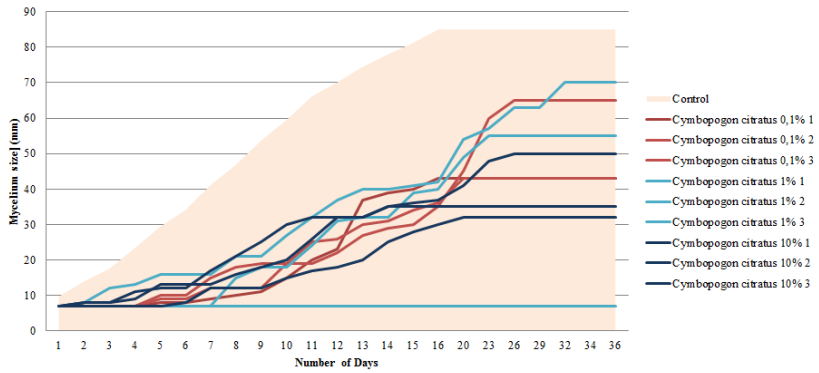

Fig. 3. Effect of *Cymbopogon citratus* EO on *Phytophthora parasitica* mycelium size at different concentrations (original)

Cymbopogon citratus EO inhibited growth at 0.1% for 4 days. At 1% concentration, the fungus showed no signs of growth, but after 7 days it started growing in 2 repetitions; in repetition no. 3 the fungus did not survive the treatment. At 10% the fungus reacted similarly to 0.1% concentration.

Since some concentrations of *Cymbopogon winterianus* and *C. citratus* EOs did not kill the microorganism, it seems it took a few days for the fungus to metabolize the constituents, transforming them in less toxic compounds. After the time requested for detoxification, the growth was no longer inhibited.

CONCLUSIONS

It is generally accepted that the treatment of microorganisms with EOs results in the impairment of membrane integrity and function. This over time may lead to the loss of cell homeostasis, the leakage of intracellular constituents and eventually cell death.

Also, these effects are generally seen in a time- and dose-dependent manner, with higher concentrations causing severe effects more rapidly and lower concentrations exerting either nonlethal effects or lethal activity only after a longer exposure time.

The common compounds of EOs that had an influence over the growth of *P. parasitica* mycelium are eugenol, eugenol acetate, iso-eugenol, borneol, dipetene, limonene and β-caryophyllene.

As the present paper showed, the oil concentrations necessary to kill *P. parasitica* are most of the times much higher than those required to inhibit their growth.

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