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Effect of essential oils on *Penicillim digitatum* growth

ERJON MAMOCI[1*], ROZETA HASALLIU[1], ENTELA HODAJ[2], MYZAFER KAPIDANI[1]

[1]Department of Agroalimentary Biotechnology, Faculty of Biotechnology and Food, Agricultural University of Tirana/ Universiteti Bujqësor i Tiranës, Kodër-Kamëz, Tiranë, Albania.

[2]Department of Biology and Chemistry, Faculty of Biotechnology and Food, Agricultural University of Tirana/Universiteti Bujqësor i Tiranës, Kodër-Kamëz, Tiranë, Albania.

Abstract

Penicillium digitatum (green mould) is one of the main pathogens of citrus fruits causing great losses during storage. Different technologies and methods are used to maintain the quality of stored citrus fruits and reduce the losses caused by green mould. Application of fungicides in pre- and postharvest is the main method to reduce losses from this pathogen. Nowadays, application of synthetic fungicides is becoming debatable because of the residues in final product and the development of resistant strains of the pathogen due to their continuous application. Natural products, such as essential oils could be used as an alternative to these fungicides aiming at partial or total replacement without having any of the above mentioned problems. In this study, different commercial essential oils from Albanian medicinal plants were tested for their activity in volatile phase against *P. digitatum*. Under *in vitro* experiments the essential oils were used at 0.28g/L air, to test the inhibition of mycelial growth. Under *in vivo* experiments the essential oils were used at 0.52g/L air to test the incidence and diameter of the lesion on artificially inoculated orange fruits. *In vitro* experiments demonstrated that essential oils of *Thymus* spp., *Origanum vulgare* and *Satureja montana* inhibited the mycelial growth of the pathogen after seven days at 24°C (total inhibition), while *Salvia officinalis*, *Laurus nobilis* and *Juniperus communis* oils promoted the growth of the fungus compared to control. The activity of all essential oils was fungistatic. In *in vivo* conditions, the tested oils of *Thymus* spp., *O. vulgare*, *S. montana* did not show inhibitory activity. However, other methods of application (contact), better oil volatization or higher doses are needed to determine their inhibitory activity. A chemical characterization of oils is also necessary to correlate it with the activity.

Keywords: ***Green mould, essential oils; antifungal, postharvest, citrus.***

1. Introduction

Green mould caused by *Penicillium digitatum* Sacc. is one of the main diseases in citrus fruits in postharvest. *P. digitatum* is a wound pathogen and has relatively short disease cycle (3-5 days at 25°C) and can produce millions of spores, which can be spread via air currents [11]. The main method to control this pathogen is the application of synthetic fungicides, such as ortho-phenylphenate, imazalil, or thiabendazole [8]. The continuous application of fungicides has given rise to resistant strains of the pathogens [11, 13] diminishing in this way their effectiveness. Also, their possible residue levels in produce and consumer perception are the main constraints to the use of synthetic fungicides.

Essential oils are an interesting strategy in the management of *P. digitatum* of fruits because they are volatile, biodegradable, harmless and more acceptable from the consumer. The main characteristic of essential oils is their activity in volatile phase as fumigants. Different essential oils have been tested against *P. digitatum* with varying degree of antifungal activity [6, 13, 24, 28].

Considering the requirements of effectiveness and convenience of the application of essential oils, there has been a constant increase in the research of alternative and efficient compounds for postharvest management aimed at partial or total replacement of synthetic chemicals. The main objective of this research was the screening of essential oils obtained by steam distillation from different Albanian medicinal plants for their antifungal activity on *P. digitatum*.

*Correspondnding author: Erjon Mamoci, E-mail: mamocie@ubt.edu.al
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2. Material and Methods

Essential oils from *Thymus* spp., *Origanum vulgare, Salvia officinalis, Laurus nobilis, Juniperus communis* and *Satureja montana* extracted by steam distillation, were furnished by "Xherdos Herbs" company Maminas, Durrës. The pathogen used in this study *P. digitatum* was isolated from infected oranges with typical symptoms of the disease (green masses of spores) on PDA (Potato dextrose agar) medium. The isolates were identified morphologically and grown in pure culture (PDA) for 5-7 days prior to *in vivo* and *in vitro* experiments. Orange fruits cv. Navel uniform in size and maturity used in *in vivo* trials were purchased in local market Kamëz, Tiranë.

2.1. In vitro antifungal activity test.

The antifungal activity of *Thymus* spp., *O. vulgare, S. officinalis, L. nobilis, J. communis* and *S. montana* essential oils were tested on colony growth using the methods given by Soylu *et al*. [25] and Shao *et al*. [20] with some modifications. Sterile plastic Petri dishes (90x15mm) with PDA medium were inoculated with mycelial discs (4mm) obtained from the edges of actively growing colony. Sterile filter paper discs (14 mm diameter) were attached to the inner surface of each Petri dish lid. The amount of 20 µl (0.28g/L) of each essential oil was added onto the filter paper, and the dishes were quickly covered. The Petri dishes were wrapped with parafilm along the rim to inhibit the release of volatile components. The compounds were allowed to volatilize inside the Petri dishes spontaneously at 24°C for three hours before the parafilm was removed. Controls were prepared similarly with the exception of the volatile treatment. Treatments were carried out with three replications. The efficacy of the treatment was evaluated by measuring the average of two perpendicular diameters of each colony.

Percentage mycelial inhibition = $[(dc - dt)/dc] \times 100$, where *dc* is the mean colony diameter for the control sets and *dt* is the mean colony diameter for the treatment sets was calculated. All tests were repeated two times.

2.2. In vivo antifungal activity test.

The antifungal activity of essential oils was tested with the methods given by Shao *et al*. [20] with some modifications. Orange fruits were surfaced-disinfected with sodium hypochlorite 2% for two minutes and washed with tap water. The fruits were artificially wounded (4x4mm diameter) in two positions. Thirty minutes later, the wounds were inoculated with mycelial discs from five-day-old cultures of *P. digitatum*. Artificially infected fruits were arranged in plastic boxes. Only essential oils from *Thymus* spp., *O. vulgaris* and *S. montana* were used at 0.52g/L air per box. The boxes without essential oil and with sterile water served as control. The boxes sealed with parafilm and essential oils were spontaneously volatilized inside the residual air space of the containers at 24°C for 3 hours and after the parafilm was removed. The incidence of decay was expressed as the percentage of infected wounds. Lesion diameter was expressed as the mean of the width and the length of each area of decay. Each treatment was replicated four times, and two fruits were used in each replicate.

2.3. Statistical analysis.

One-way analysis of variance (ANOVA) was used to determine the statistical significance; P values ≤ 0.05 were considered significant. The means were separated by Duncan's multiple range test. The data were statistically analyzed using the software package STATISTICA 6, StatSoft Inc, Tulsa, USA.

3. Results and Discussion

3.1. In vitro antifungal activity.

The colony diameter (mm) of artificially inoculated *P. digitatum* was measured after three, four, five, six and seven days on Petri dishes treated with essential oils and control (water) incubated at 24°C. Also three plates infected with mycelium of the fungus and not treated (without essential oil and water) served as control. In figure 1, the inhibition of colony diameter in percentage of essential oils is shown. Essential oils of *Thymus* spp., *O. vulgare* and *S. montana* gave 100% inhibition of mycelial growth after all the days that they were measured. Essential oils of *S. officinalis* and *L. nobilis* did not inhibit the mycelial growth, in contrary they promoted the growth of mycelium with 48.8% (82mm) and 45.1% (79.8mm) respectively, compared to control (54.7mm). The oil of *J. communis* also stimulated the mycelial growth with 28% (70mm).

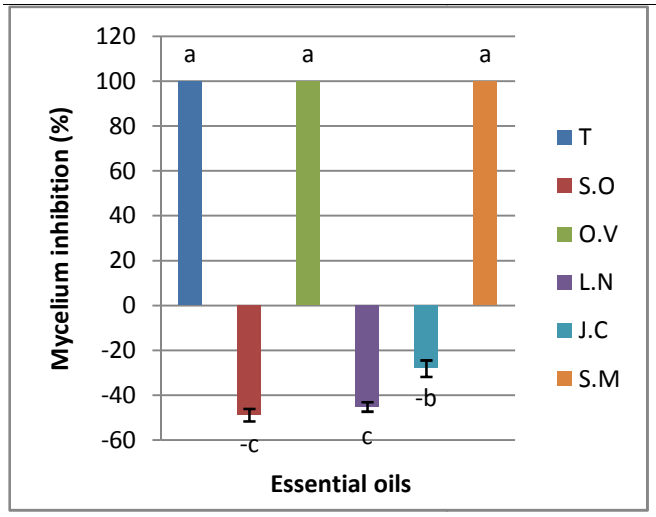

Figure 1. Inhibition of mycelial from essential oils after seven days. The negative values represent a growth higher than control. *Thymus* spp. (T), *S. officinalis* (S.O), *O. vulgare* (O.V), *L. nobilis* (L.N), *J. communis* (J.C), *S. montana* (S.M). Bars in graphic represent the mean value of three replicates (± standard error). Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test.

In figure 2, the mycelial growth treated with essential oils after seven days from inoculation is shown.

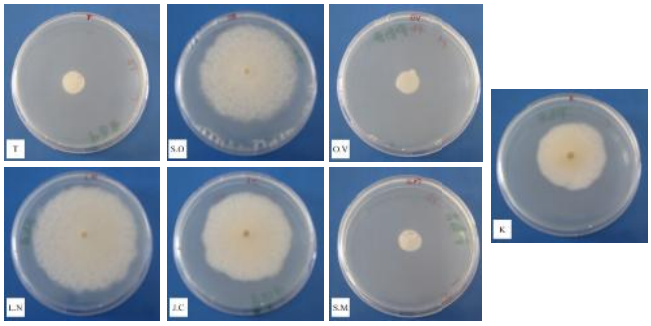

Figure 2. Growth of *P.digitatum* at 24°C treated with essential oils and control (K). *Thymus* spp. (T), *S. officinalis*(S.O), *O. vulgare* (O.V), *L. nobilis*(L.N), *J. communis* (J.C), *S. montana* (S.M).

Essential oil of *T. vulgaris* and other *Thymus* spp. have been widely studied and they are shown to be fungicidal and fungistatic. Essential oil of *T. spathulifolius* inhibited the growth of different fungi like *Fusarium* spp., *Penicillium* spp., *Rhizopus* spp., *Alternaria* spp. and *Aspergillus* spp. [23]. Acording to Klaric *et al.* [14] thymol and essential oil of *T. vulgaris* [main components *p*-cymene (36.5%) and thymol (33.0%)] gave a strong fungicidal or fungistatic activity on different species. The oil of *T. vulgaris* in volatile and contact phase affected the growth of *P. digiatum* [28]. The main components of *T. vulgaris* are α-pinene 3.3%; camphene 1.0%, β-pinene 0.6%, myrcene 1.7%, *p*-cymene 38.9%, limonene 0.8%, 1,8-cineole 1.2%, γ-terpinene 0.3%, linalool 3.8%, thymol 46.6% [7]. The antifungal activity can be classified in increasing order as: phenols > aldehydes > ketones > alcohols > esters > hydrocarbons [12]. Probably in our experiments, the main compound of *Thymus* spp. oil that inhibited the fungal growth is thymol (a phenol) confirmed also by other authors [7, 14].

The essential oil of *O. vulgare* and his main components have been described to have antimicrobial activity against many different fungal pathogens [9, 15, 27]. This oil have blocked the growth of germinative tube and altered the morphology of the hyphae of *P. digitatum* [24].

Essential oil of *S. montana* contains different compound like carvacrol, thymol, β-caryophyllene, γ-terpinene, *p*-cymene, and linalool [19]. The antimicrobial activity of this oil is attributed to phenolic compound like thymol; especially carvacrol and his precursors γ-terpinene and *p*-cymene [3, 22]. The essential oil of this plant is tested against different plant pathogens like *Alternaria solani*, *Fusarium* spp. etc [10] and postharvest pathogens like *Monilinia laxa*, *Botrytis cinerea* [16] and is shown to have antifungal activity.

The essential oil of *S. officinalis* is investigated from different authors for its antimicrobial activity. The compounds of this oil have antibacterial [26] and antifungal [1, 18] activities. In *in vitro* experiments, this oil inhibited the growth of *Alternaria alternata*, a pathogen of fruits and vegetables, in postharvest [17]. The oil altered the hyphae morphology destroying the cytoplasm and inhibited the growth of hyphae tips. In contrary to the results obtained from this authors, in our experimental conditions this oil promoted the growth of the fungus. In experiments conducted by Yahyazadeh *et al*. [28] on *P. digitatum*, this oil did not show inhibitory activity. Compounds of this oil are promoters of mycelial growth of *P. digitatum* while, they are inhibitory in other fungal species.

Simić *et al*. [21] determined that the main antifungal inhibitory compound of *L. nobilis* essential oil is 1,8-cineole. The oil of this plant obtained with CO_2 in supercritical phase is tested under *in vitro* and *in vivo* experiments on different postharvest pathogens including *P. digitatum* [6]. Only high doses of this oil give higher than 50% growth inhibition of *P. digitatum* in contact phase. In our experiments, the oil extracted with steam distillation promoted the growth of fungus but in experiments of De Corato *et al*. [6] the oil was inhibitory. These differences could be explained by the different mode of application (volatile and contact phase). Also the method of extraction could be a possible explanation since the composition may be different and this is directly correlated with activity.

The essential oil of *J. communis* has been tested under *in vitro* experiments and has shown varying degree of antifungal activity against dermatitis and species of *Candida* and *Aspergillus* [4, 5]. In experiments of Lee *et al.* [15], this oil in volatile phase did not show antifungal inhibitory activity on postharvest pathogens like *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Pythium ultimum*, *Rhizoctonia solani*. Similarly, in the present study, this oil did not inhibit the growth of *P. digitatum* in volatile phase but promoted its growth. The antifungal activity of oils may be specific; in some fungal species, it could be antifungal and in others, it could be promoter of growth or have no effect.

To determine whether the activity was fungitoxic or fungistatic, the mycelial plugs of each Petri plate was transferred after seven days in new PDA medium Petri dishes without oil. Two days later, the fungus started to grow so the essential oils of *Thymus spp.*, *O. vulgare* and *S. montana* are fungistatic in volatile phase. Since the oils of *Thymus spp.*, *O. vulgare* and *S. montana* were inhibitory under *in vitro* experiment they were tested under *in vivo* experiments.

3.2. In vivo antifungal activity.

Three days after the artificial inoculation and application of essential oils, the fruits incubated at 24°C, were evaluated for development of the disease in each wound. In figure 3, the incidence of the disease of each oil treatment is shown. Fruits treated with *Thymus* spp. and *S. montana* essential oils resulted with 68.8 and 75% infection, respectively, compared with control (62%). The essential oil of *O. vulgare* had the same value of disease incidence as control 62.5%. There were no significant differences among the treatments (P≤0.05). In *in vivo* experimental conditions (mode of application, doses used etc), the essential oils were not effective in controlling the disease incidence the fungus.

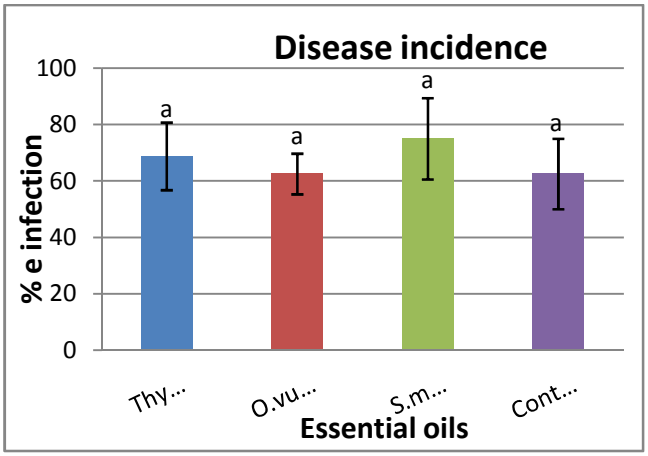

Figure 3. Disease incidence of orange fruits artificially inoculated with *P.digitatum* in presence of different essential oils and control. Each bar represents the mean percentage of 16 wounds ± standard error. Different letters indicate significant differences at *P≤ 0.05* according to Duncan's multiple range test.

The results of the diameter of the decay lesion evaluated after three days of incubation are almost similar to disease incidence (figure 4). In the fruits treated with the oil of *T. vulgaris* and *S. montana* the mean colony diameter was 26.9 and 29.1mm respectively. Both essential oils promoted the growth compared to control (22.4mm). However the oil of *O. vulgare* showed a smaller diameter lesion (20.3mm) compared with control. There were no significant differences among the treatments (P≤0.05). Therefore, in our *in vivo* experimental conditions the essential oils were not effective in controlling the lesion diameter of the fungus.

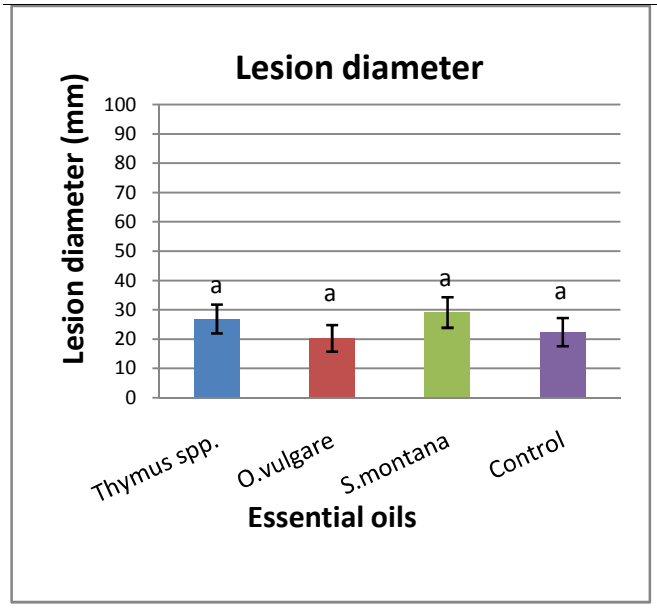

Figure 4. Lesion diameter in artificially inoculated orange fruits with *P.digitatum*, treated with different essential oils and control. Each bar represents the mean diameter of 16 wounds ± standard error. Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test.

P. digitatum is considered a wound pathogen and compounds of wounded fruits (limonene, prangolarine) promote the fungus growth [2]. The doses used in our *in vivo* experiments are not probably sufficient to inhibit the growth of the fungus in wounded fruits.

4. Conclusions

In this study the activities of different essential oils in volatile phase was tested on *P. digitatum* growth. In *in vitro* experiments essential oils of *T. vulgaris, O. vulgare, S. montana* inhibited the growth of the fungus while the other oils promoted the growth of the fungus. The antifungal activity of oils was fungistatic. The essentials oil did not show any inhibitory activity on the pathogen in *in vivo* experiments. However, other methods of application mainly contact application, better volatilization and higher doses are necessary to be studied in order to determine their activity.

5. Acknowledgements

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