ASSESSMENT OF THE ANTIFUNGAL POTENTIAL OF THE ESSENTIAL OIL FROM *Thymus vulgaris* AGAINST *Botrytis cinerea* CAUSATIVE AGENT OF POSTHARVEST GREY MOULD ON STRAWBERRY FRUITS

Mark Piero GEBEL – Franco MAGURNO

Microbiology and Environmental Toxicology Group, Institute of Plant Protection, Szent István University, H-2100 Gödöllő, Hungary
email: Franco.Magurno@mkk.szie.hu, franco.magurno@gmail.com (corresponding author), mark.gebel@hotmail.com

Abstract
Depending on commodity and region, postharvest diseases can cause considerable economic losses. Although effective against postharvest pathogens, synthetic pesticides have gained increasingly negative attention over the past years from the public opinion. Among natural fungicides, plant derived compounds such as essential oils (EOs), have become topic of increasing discussion over the last couple of decades as safe and ecologically friendly alternatives to synthetic pesticides. Grey mould caused by *Botrytis cinerea* is an important postharvest disease of strawberries and many other horticultural products. In the present study the antifungal potential of *Thymus vulgaris* essential oil was tested on individual strawberries artificially inoculated with *Botrytis cinerea.* Two dilutions of thyme essential oil, 200 µl/ml and 500 µl/ml, were applied to strawberry fruits and incubated for a period of 13 days. The dilution 500 µl/ml effectively suppressed fungal growth in strawberries for a period of 7 days, more than twice as long as the control group. Furthermore only one out of five replications in the 500 µl/ml treatment was visibly infected while the others remained free from infection for the entirety of the experiment. At experiment conclusion the strawberries in the 500 µl/ml treatment maintained a healthier shape, firmness, and colour compared to the control and 200 µl/ml treatment. This study has confirmed that the application of thyme essential oil could be an efficient biocontrol tool in postharvest stage.

Keywords: *Botrytis cinerea*, strawberry, thyme, essential oil

Introduction

Due to the perishable nature of fruits and vegetables postharvest handling presents a challenge, in particular when the commodities need to spend time in storage or transit before being available to the consumer. Depending on commodity and region, losses due to postharvest diseases can amount to as much as 50% in developing countries, representing considerable economic damages to plant products (Agrios, 2005). One of the most important factors responsible for degradation of fresh produce is decay by microorganisms, in particular by fungi (Chang et al., 2008). Infections caused during post- harvest conditions shorten the shelf life and adversely affect the market value of fruits (Tripathi et al., 2008). Therefore, most postharvest preserving agents are aimed at inhibiting or suppressing microbial growth. Physical agents, such as refrigeration and modified atmosphere, synthetic pesticides and biological control agents (BCAs) are some common practices used in the control of postharvest pathogens. Although effective, synthetic pesticides are gaining increasingly negative attention due to the concerns of health and environmental nature (Shao et al., 2013). Pesticides have been proven to disrupt the equilibrium of eco-systems, causing disease outbreak and toxicity to non-target organisms. Pesticide residues in the food are more carcinogenic compared to herbicides and insecticides and the accumulations of these residues in the food chain can sometimes reach levels above safety limits (Research Council - Board of Agriculture, 1987). Furthermore the development of pathogen strains resistant to synthetic pesticides can render them useless (Georgopoulos, 1987). To address these growing concerns the trend is to explore new ways to contrast postharvest pathogens efficiently while reducing environmental pollution and public

health risks. Much attention has been paid to the development of biological control methods of postharvest diseases in order to substitute synthetic pesticides. Some viable biological control methods used in the control of postharvest decay in fresh fruits and vegetables are application of antagonistic microorganisms such as yeasts and bacteria, the use of naturally occurring and/or non-polluting fungicidal agents (Janisiewicz and Korsten, 2002). Among natural fungicides, plant derived compounds such as essential oils (EOs), have become topic of increasing discussion over the last couple of decades. These oils are present as variable mixtures of primarily terpenoids, fatty acids, alcohols, aldehydes, aliphatic hydrocarbons, acyclic esters or lactones, coumarins and homologues of phenylpropanoids (Nazzaro et al., 2013). Extracts and oils alike of plant such as thymus, clove, cinnamon, oregano, lemongrass and many others, have all been proven to possess anti-microbial properties and are able to suppress the development of many postharvest pathogens in vitro and in vivo (Barbosa et al., 2009; Camele et al., 2012; Nuzhat and Vidyasagar, 2013). Furthermore, the use of naturally derived plant compounds, mainly essential oils, can be an efficient method to address areas where pathogens have developed resistance against pre-existing synthetic pesticides (Castillo et al., 2014).

Botrytis cinerea is an important postharvest pathogen of many horticultural crops causing major losses during storage and transport (McFeeters and McFeeters, 2012). Strawberries (*Fragaria* x *ananassa*) are very prone to infection by *Botrytis cinerea*, the causative agent of grey mould, due to their high pH, water content and the large amount of nutrients (Zamani-Zadeh et al., 2014). Due to its enormous reproduction rate by conidia and its high genetic adaptability, *Botrytis cinerea* has been classified as a high-risk pathogen with respect to fungicide resistance (Agrios, 2005) with many strains resistant to multiple (up to six) fungicides (Leroch et al., 2013).

The aim of the present study was to assess the antifungal potential of essential oil from thyme (*Thymus vulgaris*) against the grey mould on strawberry fruits. Thyme essential oil is a common product, easy to produce and cheap. It was reported by numerous investigators to have strong fungistatic and fungicidal effect on several pathogens (Bhaskara et al., 1998; Camele et al., 2012). Therefore it could be considered a valuable candidate in order to provide a postharvest protection in strawberry, where the use of synthetic pesticides is prohibited in Europe by law (Mari et al., 2014).

Materials and Methods

Plant Material

Strawberry fruits were purchased from local market (Gödöllő, Hungary) on the same day they were to be used for experimental purposes. Twenty-five berries were identified based on uniformity of size, ripeness and freedom from injury. They were surface sterilized using 2.5% sodium hypochlorite solution with exposure time of 3 minutes, washed with distilled water for 5 minutes and allowed to dry under sterile conditions.

Fungal inoculum

Botrytis cinerea strain BC4 was isolated from grape in the Plant Protection Institute of the Szent István University, Gödöllő. The strain was re-cultured on tomato-agar for two weeks at room temperature and stored at 4°C prior to use. In order to prepare the spore suspension, culture plates were washed using 0.8% Tween solution. The conidia collected were counted in a Burker chamber and the concentration was adjusted to 10^6 conidia/ml. Prior to inoculation a small wound was inflicted on the strawberry using a needle tip. Each individual berry was inoculated with 10µl of spore suspension. At experiment conclusion *Botrytis cinerea* was re-isolated from infected strawberries in order to confirm its identity under stereomicroscope at 100X magnification.

Constituent analysis and description of the essential oil

The essential oil from *Thymus vulgaris* was produced through steam distillation by Aromax

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Table 1. Constituents analysis of *Thymus vulgaris* essential oil provided by Aromax Inc.

Compound	Composition (%)
2-isopropyl-5-methlyphenol (thymol)	25 – 49.99
p-cymene	20 – 24.99
p-mentha-1,4-diene	5 – 9.99
Linalool	5 – 9.99
1,8 Cineol	2.5 - 5
p-mentha-1,3-diene	2.5 - 5
β-caryophyllene	2.5 - 5
Carvacrol	2.5 - 5
Myrcene	1 – 2.49
Camphene	1 – 2.49
α-pinene	<1
2,6-octadien-1-ol-3,7-dimethylacetate	<1
Dipentene	<1
Terpinolene	<1
3,7-dimethylocta2,6-dien-1-ole	<1
β-pinene	<1

Inc. (Budapest, Hungary). The EO was stored in a dark glass vial at room temperature. The EO dilutions with sterile distilled water were stored at 4°C prior to use. The relative composition of thyme essential oil is reported in the Table 1. The two principal constituents were thymol and p-cymene.

Experimental design

All equipment used in the experiment was disinfected using ethanol and UV radiation to minimize the presence of microorganisms foreign to the inoculum. Two dilutions of thyme essential oil in sterile distilled water, 200 µl/ml and 500 µl/ml respectively, were tested and compared with a control treatment (CON, sterile distilled water instead of EO). For each treatment five single berries as replications were placed after fungal inoculation in a 50 ml plastic container with a cellulose filter applied on the top, in a way to not come in contact with the berry. Totally fifteen berries were used in the experiment. The cellulose filters used were 1.5 cm long and 1.1 cm in diameter. A not-overflowing volume of 300 µl of EO dilution or sterile distilled water was used to saturate the filters in each container. To avoid over exposure of EO and build up of carbon dioxide in the containers the caps of the plastic containers were loosened approximately 8 hours after the inoculation and remained this way for the duration of the trial. The containers were stored at 13°C for the length of the experiment.

Evaluation of infection

Following inoculation the berries were checked every day for a period of 13 days. Documentation was carried out using a digital camera and pictures were retrieved in a standardized fashion in order to use them later for extrapolation of data. Spatial analysis was carried out using Analyzing Digital Images (ADI) suite software (http://www.umassk12.net/adi/). The diameter of the infection was measured across the inoculation point and then recorded in a database.

Statistical analysis

The statistical analysis was carried out using SPSS software version 22 (IBM). The normality of the distribution and homogeneity of variance were assessed using the Kolmogorov–Smirnov test (K–S test) and Levene test respectively. These tests were used as pre-condition tests. If p-value value from the K-S test and p-value

from the Levene test were found to be less than 0.05 the data was analyzed using the non-parametric Kruskal-Wallis test, which is better suited for asymmetric distributions and/or non-homogenous data. In addition, the Mann-Whitney U test was used as a post-hoc test for pairwise comparison between treatment groups.

Results and Discussion

In the present assay the effect of thyme oil was tested on the control of infection by *Botrytis cinerea* in garden strawberry (*Fragaria x ananassa*). Thyme oil volatiles proved to be highly effective in reducing gray mould incidence in strawberry fruits (Figure 1).

The development of infection day by day in the two EO treatments and in the control group (CON) is showed in the Figure 2. The table below the graph illustrates the average area (mm^2) of infection of each treatment during the course of the experiment. A cut-off point was put in place when the area of the infection reached 706.9 mm^2 based on the surface size of the strawberries (it was not possible to measure the diameter of infection beyond this point as the mould covered the entire surface of the strawberry).

In the control group the infection by *Botrytis cinerea* started 3 days after inoculation. In the 200 µl/ml treatment infection began on day 5 while in 500 µl/ml treatment the infection began on day 8 of the experiment. At experiment end the strawberries belonging to the 500 µl/ml treatment maintained an overall better condition than the 200 µl/ml treatment and the control group. There was some water logging in a few of the replications by the end of the experiment and clearly the fruits had become paler and lost some firmness. However, this phenomenon was less noticeable in the 500 µl/ml treatment, where half of the replications maintained a fairly health physical appearance without any browning or water soaking at all on the surface of the fruits. Out of 5 replications, in the 500 µl/ml

Figure 1. Appearance of fruits and progression of grey mold infection throughout the experiment (days: 4, 8, 13). In the figure one berry per treatment is shown as an example. Five single berries inoculated with *Botrytis cinerea* per treatments: control (berries not exposed to thyme EO), 200 µl/ml and 500 µl/ml (berries exposed to thyme EO diluted in distilled water 1:2 and 1:5 respectively)

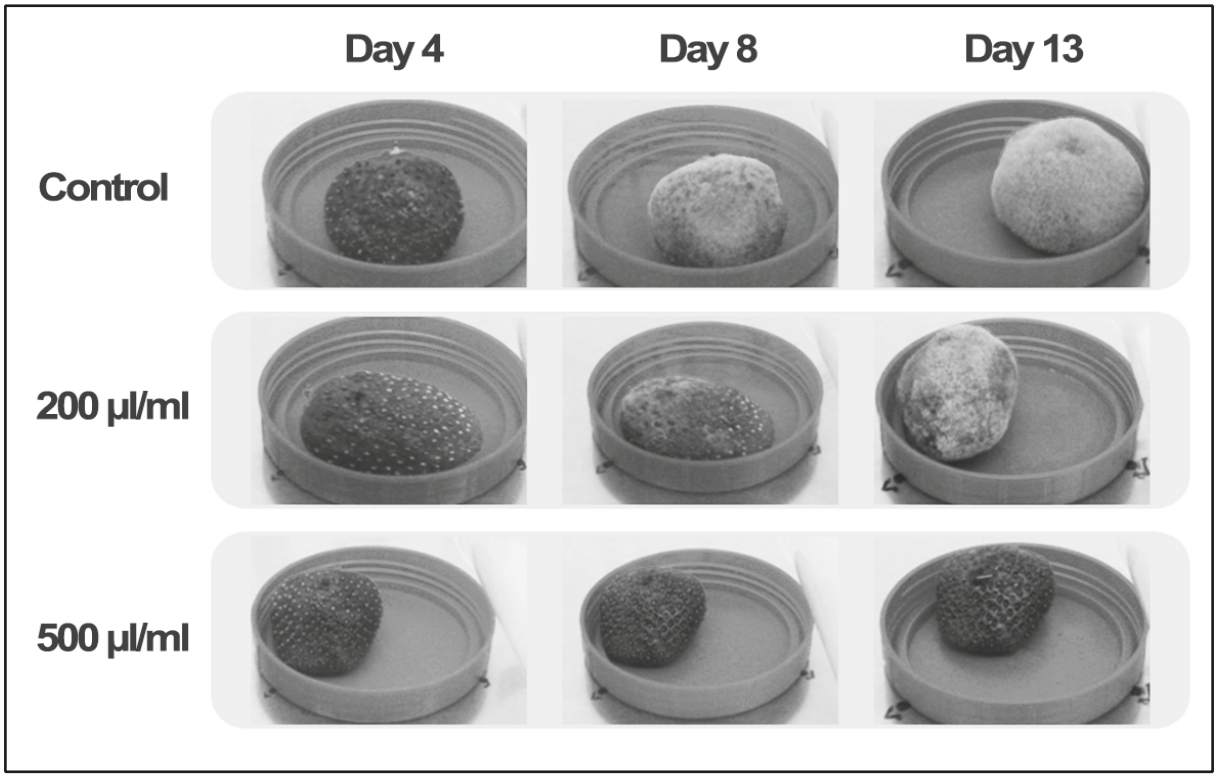

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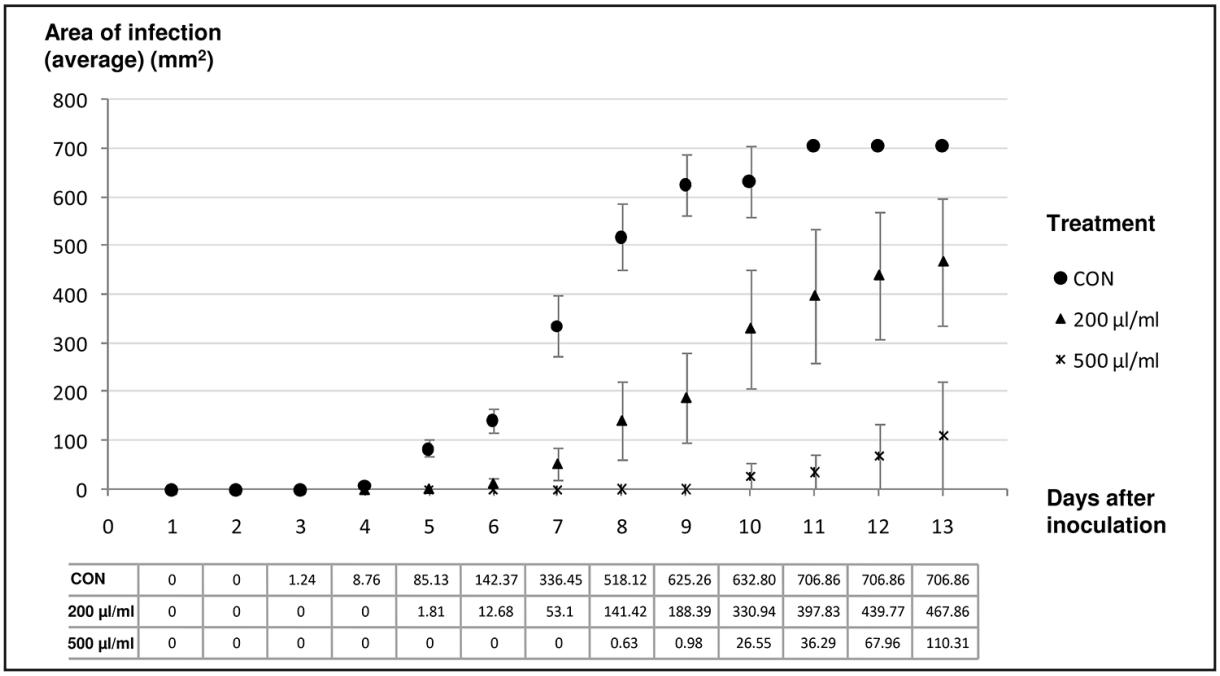
Figure 2. Development of infection by *Botrytis cinerea* in the Control treatment (CON) and in the thyme EO treatments at two dilutions (200 and 500 µl/ml). The average value of area infected in each treatment group is reported per every day of the experiment (the exact values are given in the table below the diagram). The error bars represent the standard deviation.



CON	0	0	1.24	8.76	85.13	142.37	336.45	518.12	625.26	632.80	706.86	706.86	706.86
200 µl/ml	0	0	0	0	1.81	12.68	53.1	141.42	188.39	330.94	397.83	439.77	467.86
500 µl/ml	0	0	0	0	0	0	0	0.63	0.98	26.55	36.29	67.96	110.31

treatment, only one developed mycelium on it, the others remained free from visible infection. Even though one of the replications in the 500 µl/ml treatment developed mycelium the average size of infection was approximately four times less than the 200 µl/ml treatment and seven times less than the control group at experiment conclusion (on day 13). The difference between the 200 µl/ml treatment and the control group was not very substantial. By the end of the experiment most of the berries in 200 µl/ml treatment were completely covered with grey mould. However, the effect of the oil, even though not as remarkable as in the 500 µl/ml treatment, was still visible.

After performing a pre-condition K-S test, the hypothesis of normality was rejected for all three treatment groups (including control group), with all p-values <0.05, and therefore the conditions for applying a parametric test were rejected. This was further confirmed by a second pre-condition test, the Levene test for homogeneity of variances, whose p-value was also <0.05. After performing the Kruskal-Wallis test, for non-parametric analysis of variance, a significant difference was found between the three treatment groups caused by the use of

Table 2. Kruskal-Wallis non-parametric analysis of variance test results. p-value (Asymp. Sig.) = 0.000 < 0.05; null hypothesis (all populations have identical distribution functions) is rejected

Ranks				Test Statistics	
	Treatment	**N**	**Mean Rank**		**Area**
Area	control	65	132.88	Chi-Square	60.895
	200 µl/ml	65	97.85	df	2
	500 µl/ml	65	63.26	Asymp. Sig.	.000
	Total	195			

different concentrations of EO. The p-value was <0.05 (Table 2). Since the effect of EO treatments was significant, a post-hoc Mann-Whitney U test was run on the data in order to compare between the different treatment groups. The p-values of all three comparisons performed by the Mann-Whitney U test were all <0.05 (Table 3), meaning that there was a significant effect of the presence and difference in concentration of essential oil treatment across all three groups.

In conclusion the present study demonstrated the efficiency of a commercial thyme oil volatiles in delaying significantly the occurrence of fungal infection in strawberries inoculated with *Botrytis cinerea*. The mechanism of action was not investigated but probably the oil volatiles operated reducing primarily conidial germination (Bhaskara et al., 1998). Considering the data from single replications across the different treatments (data not shown) the general trend was a significant effect of the oil volatiles on the initial development of the infection. Once the mycelium started to be visible the infection spread with an exponential rate. In particular the dilution 500 µl/ml resulted to be highly effective since the symptoms of infection were visible only in one replication on five. Our results confirmed the antifungal and food preservative properties of thyme oil observed by other authors (Bhaskara et al., 1998; Zamani-Zadeh et al., 2014). Fruits in the 500 µl/ml maintained better physical qualities such as fruit firmness and reduced water loss compared to other treatments, throughout the duration of the experiment. Furthermore, since the inoculum that was used (10^6 conidia/ml) was very concentrated it can be assumed that thyme EO tested could be a perfectly viable method to suppress postharvest development of *Botrytis cinerea*, even if a sensory quality analyses of fruits should be performed in future assays.

Table 3. Mann-Whitney U test for the pairwise comparison of the treatments: Control treatment (Con) and the thyme EO treatments at two dilutions (200 and 500 µl/ml). p-value (Asymp. Sig. (2-tailed)) = 0.000 < 0.05 in all the three comparisons; null hypothesis (populations have identical distribution functions) is rejected. According to the Z value the thyme EO treatment 500 µl/ml was found the most effective in controlling the development of the infection.

Test Statistics	**Con vs 200 µl/ml**	**Con vs 500 µl/ml**	**200 vs 500 µl/ml**
	Area	**Area**	**Area**
Mann-Whitney U	1322.500	635.000	1332.000
Wilcoxon W	3467.500	2780.000	3477.000
Z	-3.825	-7.677	-4.655
Asymp. Sig. (2-tailed)	.000	.000	.000

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