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Investigation of Growth Inhibition by Thymol and Carvacrol from *Thymus spp.* and *Origanum vulgare* on *Botrytis cinerea*

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ABSTRACT

Essential oils derived from plants in the *Lamiaceae* family, *Thymus vulgaris, Thymus capitatus,* and *Origanum vulgare,* oregano cv. 'Pierre', and their major anti-microbial volatile components carvacrol and thymol, were applied to *Botrytis cinerea* culture via headspace. In this study the components thymol and carvacrol, were adjusted to reflect their representative concentrations in the full essential oil. Growth of *Botrytis cinerea* was measured daily and treatments were subjected to ambient temperature (23°C) and cold room storage (4°C) environments. Essential oils and active antimicrobial components were applied to assess their growth inhibition activities. In Study 1, *Thymus vulgaris* essential oil, and thymol, elicited 100% growth inhibition at ambient temperature and 77% and 100% at cold room storage, respectively. In Study 2 concentrations of the essential oils of *Thymus capitatus* and *Origanum vulgare* (cv. 'Pierre'), and carvacrol showed 87%, 90%, and 100% growth inhibition at ambient temperature and 89%, 87%, and 100% inhibition at cold room storage (4°C), respectively. Results indicate that each of the three essential oils from *T. vulgaris, T. capitatus,* and *O. vulgare* are effective in inhibiting growth of *Botrytis cinerea* and suggest that the pure bioactive compounds in these essential oils are largely the cause for *Botrytis cinerea* growth inhibition. Additionally, results showed no significant differences in growth inhibition and temperature when observing impact on the reduction of growth by *B. cinerea* when using the full essential oil of Thyme and Oregano and comparing to the use of thymol and carvacrol from thyme and oregano.

INTRODUCTION

Postharvest diseases on horticultural produce account for significant loss and waste of marketable products (Alkan and Fortes, 2015). The United States losses approximately $165.6 billion dollars in food loss, with 17% accounting for produce loss, specifically (Buzby and Hyman, 2012) Further, a reported average annual loss of 11.4% of fresh fruits and 9.7% of fresh vegetables has been shown in major market retailers (Buzby et al., 2009). Given these major losses and coupled to changes in the market, largely driven by consumers, there is increasing interest in controlling these diseases with natural eco-friendly products rather than an increasing reliance on fungicide applications which have not been providing the solutions needed. These pressures have redirected research in postharvest disease mitigation to a multi-pronged approach

centered around plant secondary metabolites, more specifically, plant derived essential oils (Antunes and Cavaco, 2010)

Here, we explore the effects of *Thymus vulgaris, Thymus capitatus, and Origanum vulgare* essential oils on *Botrytis cinerea*, a well-known postharvest pathogen which had been shown to affect over 200 species of crops worldwide (Williamson et al., 2007), contributing to both vegetative and fruit loss. These three essential oils of thyme, origanum (also called wild thyme), and oregano, respectively, are part of the *Lamiaceae* family, which has been noted for antimicrobial and health benefits (Sarac and Ugur, 2007). Terpenes are the major class compounds found in these essential oils (Nieto, 2017) and are thought to be the contributing antimicrobial agents.

Thymus vulgaris and *Origanum vulgare* essential oils have been shown to be effective in inhibiting growth of *Botrytis cinerea* in culture (Hou et al., 2020; Šegvić-Klarić et al., 2007), as well as on inoculated fruit such as grapes (Martínez-Romero et al., 2007), sweet cherry (Maghenzani et al., 2018), pomegranates (Palou et al., 2016), and strawberries (Reddy et al., 1998). The major component of thyme oil, thymol, has also been shown to be effective against fungal pathogens (Šegvić-Klarić et al., 2007). Additionally, a minor component of thyme oil and isomer of thymol, carvacrol, has shown inhibitory effects on postharvest pathogens, as well (Campos-Requena et al., 2015). In *Thymus capitatus*, and *Origanum vulgare* however, carvacrol is a major compound, at 65% and 71% of the total volatile organic compounds detected, respectively.

Carvacrol and thymol have been shown to exhibit other bioactivities, such as antioxidant activity (Aeschbach et al., 1994). Carvacrol specifically has shown anti-inflammatory (da Silva Lima et al., 2013) as well as anti-ulcer (Silva et al., 2012) activities, demonstrating the potential as a multi-use additive. Thymol has also been shown to exhibit anthelmintic activity, specifically against *Haemonchus contortus,* a parasitic worm common in ruminant animals, and even anti-carcinogenic activity on promyelocytic leukemia cells (Deb et al., 2011; Jyoti et al., 2019). Though, results from previous studies show that *Thymus vulgaris*, and *Origanum vulgare* essential oils are effective against *Botrytis cinerea*, given that each of these essential oils contain many other volatile constituents, no studies have investigated if their proposed antimicrobial compounds, thymol and carvacrol, are solely responsible for eliciting these effects or to what extent these compounds contribute to the antifungal activity. Thus, the objectives of this study are to determine whether normalized concentrations of thymol and carvacrol, in *Thymus vulgaris*, or carvacrol in *Thymus capitatus* and *Orignaum vulgare*, to reflect their representative concentrations in the total oils, can elicit similar growth inhibition as the total essential oils. Apparent application of these two studies could improve value added products for industry as well as drive breeding or cultivation strategies to increase the concentrations of the most active components for a more effective essential oil.

MATERIALS AND METHODS

Chemical standards. Chemical standards were obtained from Sigma-Aldrich and are as follows: red thyme oil (*Thymus vulgaris*, MKBG9314V, 95%), origanum oil (*Thymus capitatus*: W282812), thymol (SLBH2348V), and carvacrol (MKBR5193V). Oregano essential oil, cultivar 'Pierre' (from the Rutgers breeding line OS10), was obtained via hydrodistillation (Gomes et al., 2020).

Fungal pathogen. Fungal pathogen, *Botrytis cinerea*, was isolated from grape fruit samples collected in the Northeast region of New Jersey, and identity was verified by Jeanne Peters using microscopy. Spore isolate was started on agar infused with strawberry puree, then sub-cultured onto potato-dextrose agar (PDA) plates prior to use in experiments.

Antifungal activity of thyme essential oil and monoterpenes. Relative concentrations of each essential oil, thymol, carvacrol, were calculated, and each compound was diluted to their respective reflective concentration in *Thymus vulgaris* essential oil using ethanol. The relative peak areas of thymol and carvacrol, 45%, and 5%, respectively, were determined after GC-MS analysis of *Thymus vulgaris* essential oil (red: MKBG9314V, Sigma-Aldrich). To obtain the representative concentrations, 0.13g of thymol was dissolved in 15.5uL ethanol, and 0.5uL of carvacrol was diluted with 19.5uL ethanol. A second dilution, a 1:1 dilution of each compound solution was made with ethanol, was used and 10uL of this dilution was pipetted onto filter paper (Whatman No. 8) with 2.25, and 0.25uL of thymol and carvacrol applied to the filter paper. The filter paper was then affixed to the

interior lid of a Magenta box, measuring 3"x3"x4", which had been filled with 50mL sterilized PDA. A 1-cm plug of *Botrytis cinerea* culture was placed in the center of the Magenta box. The experiment was completed in triplicate for each compound and observed at two temperatures: ambient temperature, maintained between 20-23°C and 4°C. The 4°C-temperature treatment was included to mimic the transportation environment of post-harvest fruit. Measurements were taken daily of radial growth in cm. The experiment was concluded when the control had reached 3cm, the radius of the Magenta box.

Antifungal activity of oregano essential oils and monoterpenes. Relative concentrations of carvacrol in each essential oil was calculated and diluted to its respective concentration in *Thymus capitatus* (wild thyme or origanum) and *Origanum vulgare* (cv. 'Pierre', formally known as Rutgers OS-10) essential oils using ethanol. To obtain the representative concentrations, a ratio of 1.3:1 and 1.5:1 for cv. 'Pierre' and origanum oils, for a total carvacrol application of 3.45 and 3.55uL, respectively, were applied to filter papers. Treatments were applied as described with thyme oil and component antimicrobials.

Studies 1 and 2 were completed independently of each other and at different times.

Percentage of inhibition was calculated by dividing the radial mycelial growth by the radius of the Magenta box (3cm) then multiplied by 100.

Essential oil preparation and analyses parameters. Essential oils were prepared by adding 1uL of oil to 1mL HPLC grade methyl tert-butyl ether (MtBE) and dried over sodium sulfate. The supernatant was then removed and added to HPLC vials and capped then loaded onto AOC 6000 automatic sampling tray. Essential oils were analyzed by a Shimadzu Gas Chromatograph 2010 Plus. Compounds were separated using Shimadzu SH-Rxi-5Sil column and with injection and method parameters as described in Reichert et al., (2019). A Shimadzu TQ8040 triple-Q Mass Spectrometer (MS) was used for compound identification using mass spectral libraries (NIST05.lib, NIST05s.lib, W10N14.lib and W10N14R.lib) and validated by using n-alkane standard C8-C20, to generate retention indices which were compared to published retention indices (Adams, 2007). Thymol (SLBH2348V), and carvacrol (MKBR5193V) standards were used for further identification.

Statistics: A one-way ANOVA was used to determine significance of treatments and student T-test was used to determine the presence of significance between treatments in each study. Analyses were performed using GraphPad Prism 9 software.

RESULTS

Postharvest pathogen*, Botrytis cinerea*, was subjected to *Thymus vulgaris* (thyme), *Thymus capitatus* (origanum), and *Origanum vulgare* (oregano) essential oils and their major components at a normalized concentration, with thymol 45%, and carvacrol 5%, for thyme, and 65% and 71% carvacrol for origanum and oregano oils, respectively (Table 1). The delivery mechanism for these compounds was via volatilization into headspace and their incubation took place at ambient temperature (23°C) and cold room (4°C) to observe the effect of temperature on the inhibition efficacy of the compounds. Thymol and carvacrol were the proposed antimicrobial agents with the essential oils given the studies that report their antimicrobial activity (Ben Arfa et al., 2006; Campos-Requena et al., 2015; Guarda et al., 2011; Hou et al., 2020; Martínez-Romero et al., 2007; Salehi et al., 2018; Šegvić-Klarić et al., 2007; Zhang et al., 2019). Ethanol served as the control for both studies.

Investigating thyme essential oil and thymol adjusted to be at the same concentration found in the oil, in Study 1, at both experimental temperatures, thymol and thyme oil had the highest level of growth inhibition, with 100% inhibition at ambient temperature (23°C) and 100% and 77% at cold temperatures (4°C), respectively. Likewise, carvacrol, when observed at both temperatures, showed a 40% inhibition (Table 2). Temperature differential did not play a significant role in growth. In agreement with the literature, the decrease in temperature correlates with a slowing of growth for most fungal species, hence the ubiquitous use of refrigeration for perishable foods (Kennedy et al., 2005). Ambient temperature treatments ended on Day 3 when the mycelium reached the side of the Magenta box. Cold room treatment required 6 additional days for completion compared to ambient temperature and was considered complete on day 9. The changes in the acceleration of the growth may have a significant impact on the differences in % growth inhibition (Table 2).

A one-way ANOVA indicated that there were significant ($p<0.05$) differences between the

treatments. Further, a post-hoc T-test was performed between all treatments and showed significance between carvacrol, thymol, and thyme oil ($p<0.05$), but not between thymol and thyme oil, indicating that carvacrol and the two other antimicrobial agents are not equally impactful in inhibiting the growth of *Botrytis cinerea*. However, the growth inhibition by carvacrol at ambient temperature and cold room treatments show that it still possesses effective activity in both settings.

In Study 2, we normalized the concentration of origanum and oregano (cv. 'Pierre') oil to reflect the concentration of carvacrol, and unlike the results in the thyme oil study, we observed that neither essential oils outperformed pure carvacrol, which elicited 100% inhibition at both temperatures (Table 2).

A one-way ANOVA was completed to assess whether significant ($p<0.05$) differences between the treatments existed. Further, a post-hoc T-test was performed to determine which treatments had significant differences ($p<0.05$) between one another. The ANOVA confirmed that there were significant differences in growth inhibition for both temperatures. Results of the T-test showed that there were significant differences between the control, carvacrol, and essential oils at both temperatures. When comparing the essential oils to carvacrol, no significant differences were found at cold temperature storage, but were found between oregano cv. 'Pierre' and carvacrol at ambient temperature experiments. Comparison of the essential oils between one another yielded no significant findings at either temperature. Lastly, when comparing temperature results to one another, there was also no significant difference.

The results of these two studies demonstrate the effectiveness of thymol and carvacrol in inhibiting the growth of *Botrytis cinerea*. However, it was demonstrated that carvacrol, when compared to thyme oil, did not perform as well as when it was normalized to reflect its concentration in origanum and oregano oils. Further, unlike the first study, Study 2 showed that the pure compound, carvacrol, outperformed the essential oils, indicating that there may be other compounds in the essential oil mixtures that negate the effectiveness of carvacrol. Lastly, it was demonstrated in both studies, that temperature did not significantly affect the effectiveness of either compounds or essential oils.

DISCUSSION

In Study 1, the tested concentrations of thymol, and carvacrol were adjusted to reflect the same concentrations as found in the total essential oils in *Thymus vulgaris* oil. Thymol and carvacrol, cited as antimicrobial agents in thyme essential oil (Šegvić-Klarić et al., 2007; Zhang et al., 2019), were used, but at their normalized concentrations, a previously unexplored parameter. Encapsulated thyme oil had been used to successfully mitigate the growth of *Botrytis* on strawberry. However, the method of releasing these agents is via volatilization, and a skepticism arose when it was observed that carvacrol was in such low concentration in the essential oil. This study specifically addressed the question of whether carvacrol could be acting as an antimicrobial agent in the thyme oil encapsulation system if it appears in such low concentration in the essential oil. The data shows that, when carvacrol was used at its normalized concentrations, it contributed 40% inhibition at both 4°C and ambient temperatures. The differences in these growth rates, ambient reaching completion at 3 days and 4°C at 9 days, undoubtedly a direct impact of temperature, would explain the differences in experimental completion time. Daily recordings also play a role in the disparity of the inhibition values, generating some error and creating pockets of time for more growth at one temperature versus another.

In Study 2, we again normalized the concentrations of *Thymus capitatus* (origanum) and *Origanum vulgare* (oregano) essential oils to reflect the abundance of carvacrol. These species have also been documented to inhibit the growth of *Botrytis cinerea* (Hou et al., 2020), and typically have very high concentrations of carvacrol and very little or no thymol in their oils, hence they were chosen, to compliment thyme essential oil in Study 1. In addition to normalizing their concentrations to observe whether carvacrol was their predominant antimicrobial compound, we also used a temperature differential to investigate whether temperature affected their volatilization into headspace. This study, correspondingly to Study 1, showed temperature had no significant effects on any compounds or essential oils in inhibiting the growth of *Botrytis cinerea*. Additionally, the essential oils were not shown to be significantly different indicating that though their total concentration of carvacrol, 69% and 71% carvacrol for origanum and

oregano oils, respectively, are different, it did not affect inhibition. In addition, their similar behaviors further indicates that carvacrol is their active antimicrobial compound, as they share such similar concentrations, and when observing the effect of carvacrol in Study 1, the same level of inhibition was not demonstrated.

The essential oils when compared to carvacrol, were not shown to be significantly different except with Rutgers cv. Oregano cv. 'Pierre' at ambient temperature, indicating that temperature may affect the volatilization of oregano oil at ambient temperature in such a way that it affects its inhibition. Regardless, the results of the experiment indicate that carvacrol is the antimicrobial compound in oregano and origanum oils, complimenting the results of Study 1.

Results of these experiments show that the major compound in *Thymus vulgaris* essential oil, thymol, is the principal antimicrobial compound, with carvacrol generating significant growth inhibition at ambient and 4°C storage. Likewise, carvacrol was demonstrated to be the principal antimicrobial component in *Thymus capitatus* and *Origanum vulgare*, essential oils. Additionally, the ineffectiveness of temperature on the growth inhibition capabilities of these compounds indicate that they could be used in cold storage postharvest applications, an area which *Botrytis cinerea* is often detrimental.

CONCLUSION

Our data shows that thymol is the major contributing antimicrobial agent in *Thymus vulgaris* essential oil, and carvacrol similarly in *Thymus capitatus* and *Origanum vulgare* essential oils. Carvacrol was shown to be inhibitory by 40% at ambient temperature (23°C) and cold temperatures (4°C) and elicited 100% inhibition at relative concentration in both temperatures in Study 2, suggesting that it could be used in the future in a postharvest cold storage or under refrigeration to further extend shelf-life. Future studies should address the minimum inhibitory concentrations (MIC) of carvacrol using thyme, oregano, and origanum oils to better understand the limitations this essential oil may have at different temperatures.

Table 1: Percentage composition of oregano c.v. "Pierre" (*Orignaum vulgare*), origanum (*Thymus capitatus*), and thyme (*Thymus vulgaris*) essential oils.

			Average % Peak Area		
Essential Oil Constituents	**Retention time (min)**	**Retention Index**	**Oregano oil c.v. "Pierre"**	**Origanum oil**	**Thyme oil**
Thujene	7.734	931	0.68 ± 0.0	ND*	ND*
α-Pinene	7.834	939	0.62 ± 0.0	1.30 ± 0.0	1.91 ± 0.1
Camphene	8.039	957	0.29 ± 0.0	0.26 ± 0.0	0.63 ± 0.0
1-Octen-3-ol	8.325	982	0.85 ± 0.1	0.13 ± 0.0	ND*
β-Pinene	8.357	985	1.35 ± 0.0	0.15 ± 0.0	ND*
β-Myrcene	8.436	991	2.04 ± 1.3	0.97 ± 0.0	0.72 ± 0.0
Carene	8.754	1023	1.05 ± 0.3	0.60 ± 0.0	1.50 ± 1.0
p-Cymene	8.827	1031	13.31 ± 0.0	13.99 ± 0.1	25.47 ± 0.2
Limonene	8.891	1037	ND*	0.30 ± 0.0	0.71 ± 0.0
Eucalyptol	8.937	1042	ND*	0.31 ± 0.0	0.58 ± 0.2
γ-Terpinene	9.164	1066	3.09 ± 0.1	1.09 ± 0.0	3.08 ± 0.0
p-Mentha-3(8), 6-dien	9.427	1093	ND*	ND*	0.68 ± 0.0
Linalool	9.508	1102	0.23 ± 0.0	2.00 ± 0.0	4.57 ± 0.0
Unknown	9.567	1109	0.18 ± 0.0	ND*	ND*
endo-Borneol	10.204	1186	0.88 ± 0.0	0.45 ± 0.0	0.57 ± 0.0
Terpinen-4-ol	10.254	1192	0.30 ± 0.0	0.83 ± 0.0	0.55 ± 0.0
α-Terpineol	10.358	1205	ND*	0.33 ± 0.0	1.93 ± 0.0
Thymol	11.023	1293	0.27 ± 0.0	9.30 ± 0.0	45.21 ± 1.4
Carvacrol	11.102	1303	71.40 ± 1.0	65.70 ± 0.2	5.33 ± 0.1
Unknown	11.969	1429	ND*	ND*	0.67 ± 1.0
Unknown	12.033	1438	ND*	ND*	1.57 ± 1.5
Caryophyllene	12.096	1448	0.40 ± 0.0	1.49 ± 0.0	2.64 ± 0.0
β-Bisabolene	12.565	1520	0.74 ± 0.0	ND*	ND*
Caryophyllene oxide	13.15	1614	1.50 ± 0.1	0.81 ± 0.0	ND*
Unknown	13.314	1640	0.22 ± 0.0	ND*	ND*
Unknown	15.634	2012	0.62 ± 1.1	ND*	ND*

* ND; not detected; ± = standard deviation between samples

Table 2: Inhibition of *Botrytis cinerea*, using thyme (*Thymus vulgaris*) essential oil, and constituents and origanum (*Thymus capitatus*) and oregano c.v. "Pierre" (*Origanum vulgare*) essential oils, respectively.

	Average Growth Inhibition (%)	
Essential Oils and Single Aromatic Oil Constituents	**4°C**	**Ambient (23°C)**
Study 1		
Thyme Oil (*Thymus vulgaris*)	100±0.0	77.7±0.16
Thymol	100±0.0	100±0.0
Carvacrol	40±0.33	40±0.23
Control	0±0.0	0±0.0
Study 2		
Oregano c.v. "Pierre" (*Origanum vulgare*)	87± 0.17	90±0.11
Origanum (*Thymus capitatus*)	89±0.13	87±0.17
Carvacrol	100±0	100±0
Control	0±0	0±0

*± = the standard error of replicated for each treatment; Growth Inhibition = the reduction of *Botrytis cinerea* growth was calculated by comparing control treatments, where 0% inhibition was the reaching of mycelia to the edge of the magenta box, to the radial growth of treatments.

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