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Co-treatment with Origanum Oil and Thyme Oil Vapours Synergistically Limits the Growth of Soil-borne Pathogens Causing Strawberry Diseases

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Vapours from origanum oil (O) and thyme oil (T) were applied to the four soil-borne strawberry pathogens *Fusarium oxysporum* f. sp. *fragariae*, *Colletotrichum fructicola*, *Lasiodiplodia theobromae*, and *Phytophthora cactorum*, causing Fusarium wilt, anthracnose, dieback, and Phytophthora rot, respectively. Increasing T vapour doses in the presence of O vapour strongly inhibited mycelial growths of the four pathogens and vice versa. When mycelia of *F. oxysporum* f. sp. *fragariae* and *P. cactorum* exposed to the combined O + T vapours were transferred to the fresh media, mycelial growth was restored, indicating fungistasis by vapours. However, the mycelial growth of *C. fructicola* and *L. theobromae* exposed to the combined O + T vapours have been slightly retarded in the fresh media. Prolonged exposure of strawberry pathogens to O + T vapours in soil environments may be suggested as an alternative method for eco-friendly disease management.

Keywords **:** antimicrobial, plant essential oil vapours, strawberry diseases

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Different diseases in strawberry nurseries and fields have devastated strawberry fruit production worldwide. Several strawberry diseases are caused by soil-borne fungal pathogens *Fusarium oxysporum* f. sp. *fragariae* and various *Colletotrichum* spp. such as *C. fragariae*, *C. fructicola*, and *C. gloeosporioides* (MacKenzie et al., 2008; Nam et al., 2005, 2013; Ureña-Padilla et al., 2002). Oomycete pathogens *Phytophthora cactorum*, *P. fragariae*, and *P. nicotianae* causing severe symptoms could infect different strawberry plant organs through the soil (Bonants et al., 1997; Li et al., 2013). Recently, dieback and black rot on runners and daughter plants of strawberry seedlings (cv. Seolhyang) have been found in nursery fields in South Korea (Nam et al., 2016). It was the second report on the strawberry disease by *Lasiodiplodia theobromae* after the first reported disease occurrence in Turkish strawberry fields (Yildiz et al., 2014). Since it has been known that *L. theobromae* usually invades the many woody plants such as grapevines, mango, mulberry, and peach, strawberry seedlings showing dieback and black rot can be worrying about the disease spreading on herbaceous plants trees (Gava et al., 2022; Gnanesh et al., 2022; Li et al., 2019; Úrbez-Torres and Gubler, 2011). There is an increasing concern about dieback and black rot diseases on the strawberry seedlings because cv. Seolhyang is prevalent in South Korea and is highly susceptible to *L. theobromae* inoculation (Nam et al., 2016).

Conventional chemical controls using synthetic fungicides to deal with strawberry fungal diseases sometimes have led to the occurrence of fungicide-resistant isolates in the strawberry fields (Forcelini et al., 2016; Koike and Gordon, 2015; Luo et al., 2021; Marin et al., 2021). Eco-friendly and sustainable alternatives for chemical fungicides have been evaluated. Various antagonistic bacteria (*Bacillus velezensis*, *Pseudomonas fluorescence*, and *Streptomyces* spp.) and fungi (*Aureobasidium pullulans* and *Cladophia-*

lophora chaetospira), have been employed for eco-friendly biological control of strawberry diseases (Agustí et al., 2011; Harsonowati et al., 2020; Iqbal et al., 2021; Marian et al., 2020; Nam et al., 2009). The application of plant essential oils or their chemical components was also suggested as one of the plant disease control methods for organic horticultural crop production and stable food storage (Chang et al., 2022; Sivakumar and Bautista-Baños, 2014). Pouring diluted clove oil onto commercial soil mixture in pots significantly delayed the bacterial wilt of tomato seedlings inoculated by *Ralstonia peudosolanacearum* (Lee et al., 2012). Treatment with cinnamon oil vapour reduced lesion formation in *Alternaria alternata*-inoculated tomato leaves and *C. gloeosporioides*-inoculated pepper fruits (Hong et al., 2015, 2018).

Diverse plant essential oils were widely used to manage strawberry diseases as well. Anthracnose on strawberry fruits by *C. nymphaeae* infection was reduced by plant essential oils from dill seed, garlic, rosemary, lavender, lemongrass, and sage (Hoseini et al., 2019; Hosseini et al., 2020; Karimi et al., 2016). *In vitro* antimicrobial activity of plant essential oils was demonstrated against several *Phytophthora* spp. infecting different host plants (Amini et al., 2016; Diánez et al., 2018; Soylu et al., 2006). But it is hard to find the inhibitory effect of plant essential oils on growths of *P. cactorum*, *P. nicotianae*, or *P. fragariae* causing strawberry diseases. Plant essential oils inhibiting *in vitro* growth of *L. theobromae* have been evaluated, but using plant essential oils-based disease control was only applied in mango fruits against stem end rot (Combrinck et al., 2011; Perumal et al., 2016). In our previous study, inhibitory activities of four plant essential oils (cinnamon oil, fennel oil, origanum oil, thyme oil) were investigated against *in vitro* fungal growth of *F. oxysporum* f. sp. *fragariae*, a causal agent of Fusarium wilt in strawberry plants (Park et al., 2017). In particular, co-treatments of *F. oxysporum* f. sp. *fragariae* mycelia with origanum oil and thyme oil vapours have shown synergistic antifungal activities. In this study, dose-dependent synergistic antimicrobial activities of co-treatments with origanum oil and thyme oil vapours against the *F. oxysporum* f. sp. *fragariae,* as well as other soil-borne phytopathogens causing anthracnose, dieback, and Phytophthora crown rot in strawberry plants to investigate whether the co-treatment can be applied for simultaneous controls of the four diseases in the strawberry fields.

Synergistic antimicrobial activities of origanum oil and thyme oil vapours were investigated against the four soil-borne strawberry pathogens (Fig. 1). For each plant essential oil vapour treatment, one paper disc harbouring

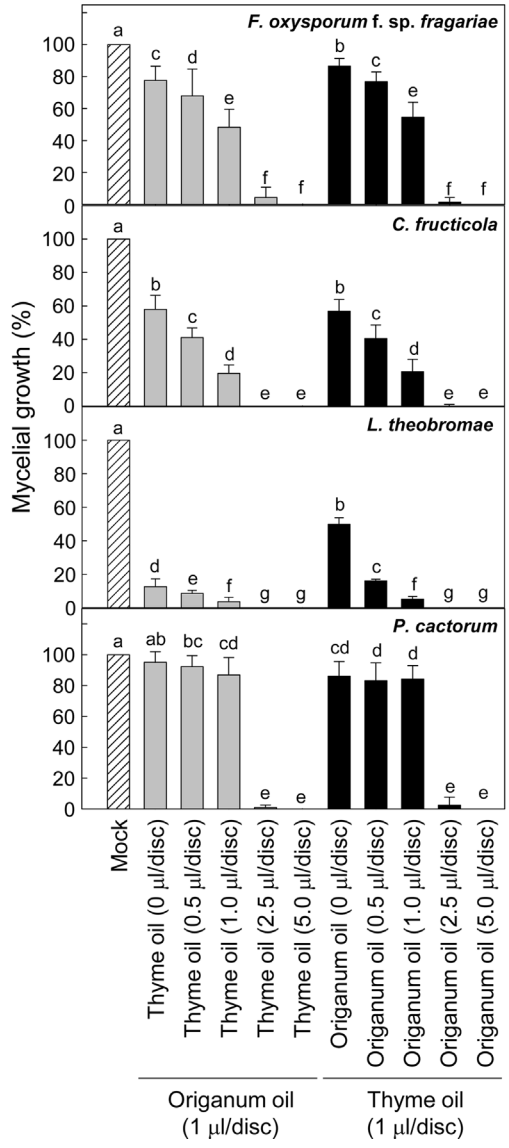

Fig. 1. Relative mycelial growth of four pathogens causing strawberry diseases, Fusarium wilt, anthracnose, dieback and Phytophthora rot by co-treatment with different doses of origanum oil and thyme oil vapours. Colony diameters of *Fusarium oxysporum* f. sp. *fragariae* isolate SFW-10, *Colletotrichum fructicola* isolate SAn-3, *Lasiodiplodia theobromae* isolate LT120902 and *Phytophthora cactorum* isolate P 9815 (KACC 40183) on the 1/2-strength potato dextrose agar (PDA) media, were measured at 7, 5, 2 and 10 days after cultures at 25°C, respectively. Colony formation on the PDA media arrested by the two plant essential oil vapours compared to a colony in mock-treated control are demonstrated as the relative mycelial growths (%). Error bars represent standard errors of means of five independent experimental replications. Each experiment contained four biological replicates. Means followed by the same letter are not significantly different at the 5% level by the least significant difference test. The same letter above bars represented no significant difference between treatments.

each plant essential oil dose was attached inside the Petri dish covers as described before (Park et al., 2017). For the combined application of the origanum oil and thyme oil vapours, one paper disc containing one essential oil dose (1 µl/disc) was attached simultaneously with one paper disc having increasing amounts of another essential oil (0, 0.5, 1.0, 2.5, and 5 µl/disc). *F. oxysporum* f. sp. *fragariae* isolate SFW-10, *C. fructicola* isolate SAn-3, *L. theobromae* isolate LT120902 and *P. cactorum* isolate P 9815 (KACC 40183) were inoculated on the 1/2-strength potato dextrose agar medium using mycelial discs (5 mm in diameter) and cultured for 7, 5, 2, and 10 days at 25°C, respectively.

Vapour from origanum oil (1 µl/disc) alone was enough to decrease the mycelial growth of *F. oxysporum* f. sp. *fragariae* compared to the mock-treatment (ca. 78%). Increasing vapour doses of thyme oil (0.5, 1, 2.5, and 5 µl/disc) in the presence of origanum oil (1 µl/disc) dose-dependently decreased the mycelial growth to ca. 68%, 48%, 4%, and 0%. Vapour from thyme oil (1 µl/disc) alone also could reduce mycelial growth (ca. 87%). Increasing vapour doses of origanum oil (0.5, 1, 2.5, and 5 µl/disc) in the presence of thyme oil (1 µl/disc) gradually reduced the mycelial growth to ca. 77%, 55%, 1%, and 0% in a dose-dependent manner. Either origanum oil (1 µl/disc) or thyme oil (1 µl/disc) alone decreased the mycelial growth of *C. fructicola* by the statistically same levels. Vapours of thyme oil (0.5 and 1 µl/disc) in the presence of origanum oil (1 µl/disc) showed more excellent antifungal activity against the mycelial growth of *C. fructicola*, showing 41% and 20% compared to the mock-treatment, respectively. The more vapours from thyme oil (2.5 and 5 µl/disc) with vapour from origanum oil (1 µl/disc) completely suppressed the mycelial growth. The antifungal activity against *C. fructicola* was enhanced by higher vapours of origanum oil (0.5 to 5 µl/disc) in the presence of thyme oil (1 µl/disc), showing ca. 40%, 21%, 0%, and 0% mycelial growths compared to the mock-treatment. *L. theobromae* was highly vulnerable to vapour from thyme oil. Treatment with vapour from thyme oil (1 µl/disc) drastically suppressed the mycelial growth of *L. theobromae* compared to the mock-treatment (ca. 12%). The reduced mycelial growth was more limited to ca. 9%, 4%, 0%, and 0% by the co-treatment with vapour from thyme oil (1 µl/disc) and vapours from increasing origanum oil (0.5, 1, 2.5, and 5 µl/disc). Vapour from origanum oil (1 µl/disc) also impeded the mycelial growth of *L. theobromae* (ca. 50%). Vapours from increasing thyme oil (0.5, 1, 2.5, and 5 µl/disc) cooperatively enhanced the antifungal activity of vapour from origanum oil (1 µl/disc) with ca. 16%, 5%, 0%, and 0%. Mycelial growth of *P. cactorum* was not arrested by vapour from origanum oil (1 µl/disc) alone. Additional vapours from thyme oil (0.5 and 1 µl/disc) slightly reduced the mycelial growth to 89% and 83%, respectively, compared to the mock-treatment. Supplementing with vapours from higher thyme oil doses (2 and 5 µl/disc) with vapour from origanum oil (1 µl/disc) completely inhibited the mycelial growth of *P. cactorum*. Vapour from origanum oil (1 µl/disc) alone showed low antimicrobial activity against the mycelial growth of *P. cactorum* (ca. 82%). Additional vapours from thyme oil (0.5 and 1 µl/disc) could not improve the antimicrobial activity. No mycelial development of *P. cactorum* was found by vapour from origanum oil (1 µl/disc) together with vapours from higher doses (2 and 5 µl/disc). Mycelial sensitivities of the four pathogens differed against the same vapour combination treatments. However, current results showed that combined treatment with vapours from higher doses of origanum oil and thyme oil could increase antimicrobial activities against the four strawberry pathogens. Synergistic antifungal activities of plant essential oils have been evaluated for food protection against fungal spoilage (Hossain et al., 2016; Nikkhah et al., 2017). Interestingly, the oregano and thyme oil combination exhibited the highest antifungal activity against *Aspergillus niger*, *A. flavus*, *A. parasiticus*, and *Penicillium chrysogenum,* among other plant essential oil combinations including basil, cinnamon, eucalyptus, mandarin, oregano, peppermint, tea tree and thyme oils (Hossain et al., 2016). A mixture of two or more essential oils can be applied as an effective disinfectant to the soil and medium in strawberry nurseries and fields prior to planting. It will simultaneously reduce the inocula of the four pathogens in the soils or media. We should further carefully evaluate the enhanced antimicrobial activities using the combined plant essential oil vapours because strawberry plants at different growth stages can be damaged by the combined plant essential oil vapours. Plant essential oil vapours or volatile residues can cause adverse effects on plant growth and development (Han et al., 2021; Silva et al., 2021; Zhou et al., 2021). Therefore, the use of essential oils during strawberry plant cultivation requires a detailed investigation of their phytotoxic effects.

Viablity of the four strawberry pathogens was investigated after treatment with the combined vapours (O + T) from origanum oil (1 µl/disc) and thyme oil (5 µl/disc) followed by the vapour removal (Fig. 2). Fungal colonies of *F. oxysporum* f. sp. *fragariae* were cultured for 3 days at 25°C, and then O + T vapours were treated to the fungal cultures for 1 or 3 days. Mycelial growth of *F. oxysporum* f. sp. *fragariae* was reduced at 1 and 3 days after the O + T vapour treatment compared to the mock-treatment (Fig.

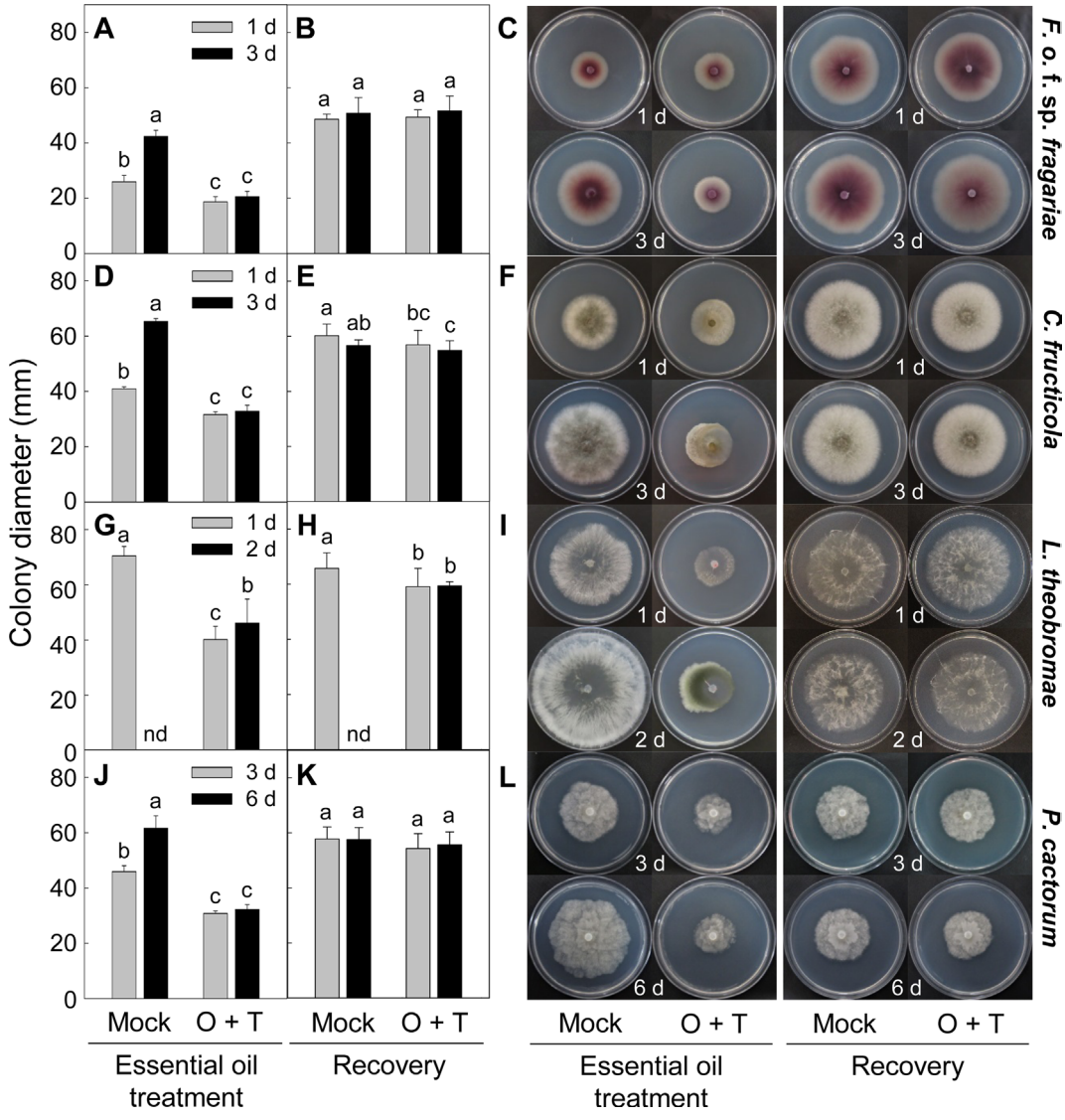

Fig. 2. Mycelial growth of four pathogens causing strawberry diseases, Fusarium wilt, anthracnose, dieback and Phytophthora rot, suppressed by co-treatment with origanum oil (1 µl/disc) and thyme oil (5 µl/disc) vapours (O + T) and rescued by transfer to the fresh media in the absence of the vapours. Colony diameters (mm) of *Fusarium oxysporum* f. sp. *fragariae* isolate SFW-10 (A), *Colletotrichum fructicola* isolate SAn-3 (D), *Lasiodiplodia theobromae* LT120902 (G), and *Phytophthora cactorum* isolate P 9815 (J) (KACC 40183) on the 1/2-strength potato dextrose agar (PDA) media were measured by mock and O + T treatments at different exposure times: grey and black bars within the graphs indicate two exposure periods to the combined O + T essential oil vapour treatment. Mycelial plugs from the margin of essential oil vapour-treated colonies were transferred to 1/2-strength PDA media and cultured for 7, 5, 2 and 10 days after cultures at 25°C, for *F. oxysporum* f. sp. *fragariae* (B), *C. fructicola* (E), *L. theobromae* (H), and *P. cactorum* (K), respectively. Error bars represent standard errors of means of five independent experimental replications. Each experiment contained four biological replicates. Means followed by the same letter are not significantly different at the 5% level by the least significant difference test. The same letter above bars represented no significant difference between treatments. nd, not determined. Colony morphologies of *F. oxysporum* f. sp. *fragariae* (C), *C. fructicola* (F), *L. theobromae* (I), and *P. cactorum* (L) were observed before and after rescue from essential oil vapour treatments.

2A). Mycelial discs were cut from the growing edge of the fungal cultures with or without O + T vapour treatment for subsequent cultures in a new media. There was no difference between the mock and O + T vapour treatment in the mycelial growth of *F. oxysporum* f. sp. *fragariae* at 7 days after recovery in the new media (Fig. 2B). *C. fructicola* colonies grown for 3 days at 25°C were treated with O + T vapour for 1 or 3 days. The mycelial growths of *C. fruc-*

ticola were significantly decreased by the O + T vapour compared to the mock-treatment (Fig. 2D). *C. fructicola* grew well in the new media after 5 days after recovery, but mycelial growths of the O + T vapour-treated *C. fructicola* were still retarded and significantly reduced (Fig. 2E). One-day old culture colonies of *L. theobromae* were exposed to the O + T vapours for 1 or 2 days. The O + T vapour-treated *L. theobromae* were subcultured in fresh media for 2 days after removing the vapours. The mycelial growths of *L. theobromae* were suppressed by the O + T vapour at 1 day compared to the mock-treatment (Fig. 2G). We could not determine the effect of 2 day-exposure to the O + T vapour on the mycelial growths of *L. theobromae,* because 1-day cultured fungal colonies of *L. theobromae* were fast grown and reached inside the Petri dishes after an additional 2 day-culture in the absence of the O + T vapour. *P. cactorum* colonies cultured for 5 days at 25°C were treated with the O + T vapour for 3 or 6 days, and the colonies were additionally subcultured for 10 days in the absence of the vapour. The mycelial growths of *P. cactorum* were arrested in the presence of the O + T vapours compared to the mock-treatment (Fig. 2J), but we could not find the mycelial growth limitation of *P. cactorum* in the fresh media after removal of the O + T vapours compared to the mock-treatment (Fig. 2K). Representative cultures of the four strawberry pathogens *F. oxysporum* f. sp. *fragariae*, *C. fructicola, L. theobromae,* and *P. cactorum* with or without O + T vapour treatment and recovery were demonstrated in Fig. 2C, F, I, and L, respectively. These results indicate that O + T vapour-mediated microbial growth reduction occurred in relatively short periods can be restored by loss of the O + T vapour.

Confilicts of Interest

No potential conflict of interest to this article was reported.

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