Differential Inhibitory Activities of Four Plant Essential Oils on In Vitro Growth of Fusarium oxysporum f. sp. fragariae Causing Fusarium Wilt in Strawberry Plants

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(Received on June 30, 2017; Revised on August 16, 2017; Accepted on August 21, 2017)

The objective of this study was to determine inhibitory activities of four volatile plant essential oils (cinnamon oil, fennel oil, origanum oil and thyme oil) on in vitro growth of Fusarium oxysporum f. sp. fragariae causing Fusarium wilt of strawberry plants. Results showed that these essential oils inhibited in vitro conidial germination and mycelial growth of F. oxysporum f. sp. fragariae in a dose-dependent manner. Cinnamon oil was found to be most effective one in suppressing conidial germination while fennel oil, origanum oil and thyme oil showed moderate inhibition of conidial germination at similar levels. Cinnamon oil, origanum oil and thyme oil showed moderate antifungal activities against mycelial growth at similar levels while fennel oil had relatively lower antifungal activity against mycelial growth. Antifungal effects of these four plant essential oils in different combinations on in vitro fungal growth were also evaluated. These essential oils demonstrated synergistic antifungal activities against conidial germination and mycelial growth of F. oxysporum f. sp. fragariae in vitro. Simultaneous application of origanum oil and thyme oil enhanced their antimicrobial activities against conidial germination and fungal mycelial growth. These results underpin that volatile plant essential oils could be used in eco-friendly integrated disease management of Fusarium wilt in strawberry fields.

Keywords : antifungal, Fusarium oxysporum f. sp. fragariae, plant essential oil, synergistic, volatile

Handling Associate Editor : Jeon, Junhyun

Fusarium wilt by Fusarium oxysporum is one intractable soil-borne plant disease that causes devastating losses of many economically important horticultural crops, including banana, muskmelon, tomato and strawberry plants. Fusarium wilt has been continuously spreading in strawberry plant growing fields. Strawberry Fusarium wilt disease has been increasing reported in many countries in Europe, Asia and America recently (Arroyo et al., 2009; Dinler et al., 2016; Koike et al., 2009; Stanković et al., 2014). In South Korea, strawberry Fusarium wilt occurs in all plant growing seasons, including nursery and protected cropping field stages of several cultivars prevalently planted such as Akihime and Maehyang (Nam et al., 2005). Genetic diversity has been found in Korean isolates of F. oxysporum f. sp. fragariae depending on strawberry cultivation regions (Kim et al., 2017).

To control strawberry Fusarium wilt, a variety of approaches have been suggested, including cultural, biological and chemical methods. Planting disease-resistant cultivars against Fusarium wilt is the most promising approach for sustainable horticulture. Commercial strawberry cultivars resistant to Fusarium wilt have been analysed (Cho and Moon, 1984; Fang et al., 2012; Kim et al., 1982; Mori et al., 2005; Nam et al., 2005). Strawberry cultivar highly

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resistant to Fusarium wilt has been hardly reported worldwide. Cultivar Seolhyang is relatively resistant to Fusarium wilt compared to cultivar Maehyang (Lee et al., 2010). Cultivar Seolhyang is planted in more than 70% of strawberry fields in South Korea. This is dangerous because highly virulent isolates can overcome cultivar resistance. Although in vitro inhibitory effects of several chemical fungicides such as thiophanate-methyl on growth of *Fusarium oxysporum* f. sp. *fragariae* have been investigated, only limited number of these fungicides such as copper hydroxide have been used to control strawberry Fusarium wilt in fields (Cho and Moon, 1984; Kim et al., 1982). Supplement with organic matter and calcium cyanide during soil solarization can reduce Fusarium wilt before transplanting strawberry seedlings into fields (Nam et al., 2011). Eco-friendly biocontrol agents are needed to introduce strawberry plant cultures during growing seasons. In recent years, antagonistic bacteria (*Bacillus velezensis*, *Streptomyces* sp.) and symbiotic mycorrhizal fungus (*Glomus mosseae*) have been applied in strawberry growing fields to decrease Fusarium wilt (Cha et al., 2016; Matsubara et al., 2004; Nam et al., 2009). Recent progresses in management strategies of strawberry Fusarium wilt have been reviewed by Koike and Gordon (2015).

Plant essential oils with a broad spectrum of antimicrobial activity have been applied to manage fungal and bacterial diseases of many vegetables. They might be used as green pesticides in integrated pest management programmes (Bajpai et al., 2011; Koul et al., 2008; Sivakumar and Bautista-Baños, 2014). Our previous studies have shown that different plant essential oils can efficiently control pepper fruit anthracnose caused by *Colletotrichum gloeosporioides* fungus and tomato plant wilting caused by *Ralstonia pseudosolanacearum* bacterium (Hong et al., 2015; Lee et al., 2012). Applying plant essential oils has been strongly suggested as an alternative method to control Fusarium wilt of banana, muskmelon and tomato plants (Bowers and Locke, 2000; Monteiro et al., 2013; Sharma et al., 2017). However, using plant essential oils to control Fusarium wilt of strawberry has not been reported yet.

Therefore, the objective of this study was to evaluate inhibiting activities of four plant essential oils (cinnamon oil, fennel oil, origanum oil and thyme oil) on in vitro growth of *F. oxysporum* f. sp. *fragariae*. These four plant essential oils have been reported to possess antifungal activities against conidial germination and/or mycelial germination of a variety of phytopathogenic fungi at relatively low concentrations (Daferera et al., 2003; Monteiro et al., 2013; Soylu et al., 2007, 2010). In addition, synergistic effect of these plant essential oils in combination on in vitro fungal growth was also evaluated in this study.

**Materials and Methods**

**Fungal cultures.** *F. oxysporum* f. sp. *fragariae* isolate SFW-10 used in the present study was isolated from naturally infected strawberry plants (cv. Akihime) grown in Hadong-gun, Gyeongnam, South Korea in 2010. This fungus was cultured on 1/2 potato dextrose agar (PDA) media at 25°C. To obtain conidial suspension, mycelia were streaked onto culture media and incubated for 14 days. Quarterly diluted sterile potato dextrose broth (PDB) was poured onto the 14 day-old fungal culture. The concentration of conidial suspension was adjusted to 5 × 10⁷ conidia/ml. To prepare mycelial plugs, mycelial discs (5 mm in diameter) were inoculated at the center of the media. Fresh mycelial discs (5 mm in diameter) were cut from the growing edge of 7 day-old PDA cultures.

**Preparation of paper disc harbouring plant essential oils.** Cinnamon oil and thyme oil were purchased from Sigma-Aldrich Co., Ltd (St. Louis, MO, USA). Fennel oil and origanum oil were purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Each viscous plant essential oil concentrate was diluted to 5 or 20% (v/v) solution in 95% ethanol for easy handling. Increasing doses of each plant essential oil (0, 0.1, 0.2, 0.5, 1, 2 and 5 µl/disc) were dropped onto sterile paper disc (8 mm in diameter) (Toyo Roshi Kaisha, Ltd, Tokyo, Japan) and 95% ethanol was additionally laid to make the same loading volume (50 µl) in each paper disc. These paper discs were air-dried for 20 min at room temperature to evaporate ethanol on a clean bench.

**Inhibitory effect of volatile plant essential oils on in vitro conidial germination and mycelial growth.** Volatile antifungal activities of these four plant essential oils against in vitro conidial germination and mycelial growth were evaluated using method described in our previous study (Hong et al., 2015) with slight modifications. Briefly, for conidial germination-inhibiting assays, four conidial suspension drops (20 µl each) prepared in 1/4 PDB on glass slides were treated with plant essential oils for 10 h at 25°C in plastic square dishes (100 mm × 100 mm × 13 mm). Conidia were stained with 1 µl of lactophenol-trypan blue solution and conidial suspensions were covered by coverslips before observation under a microscope. For mycelial growth-inhibiting assays, fungal mycelial plugs (5 mm in diameter) were inoculated onto the centre of 10 ml of PDA media in Petri dishes (85 mm in diameter, 13 mm
in height). Fungal colony diameters were measured at 6 days after inoculation onto PDA media at 25°C. Diameters of fungal colonies treated with plant essential oils were compared to those of untreated colonies and expressed as percentage (%).

**Simultaneous treatment with different plant essential oils.** Different plant essential oils were applied simultaneously to determine their synergistic efficacies against *in vitro* conidial germination and mycelial growth of *F. oxysporum* f. sp. *fragariae*. Paper discs containing 0.5 and 1 µl/disc of each plant essential oil were attached to the inside plate cover singly or concurrently with other paper disc(s) for conidial germination and mycelial growth inhibition assays, respectively. Conidial germination and mycelial growth were evaluated at 10 h and 6 days after incubation at 25°C with different combinations of these plant essential oils described above.

**Statistical analyses.** Data were subjected to analysis of variance using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA). Means were separated by least significant difference (LSD) test at *P* < 0.05. Graphing was prepared using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

**Results**

**Different antifungal efficacies of four plant essential oils against *in vitro* conidial germination of *F. oxysporum* f. sp. *fragariae*.** The four plant essential oils tested in this study showed different antifungal efficacies against *in vitro* conidial germination of *F. oxysporum* f. sp. *fragariae* in dose-dependent manners (Fig. 1). Treatment with cinnamon oil drastically suppressed conidial germination at the lowest dose used (0.1 µl/disc). Conidial germination was completely inhibited by cinnamon oil at 0.2 µl/disc. Fennel oil was less effective in reducing conidial germination compared to cinnamon oil. It failed to completely suppress conidial germination even at the highest dose used in this study (5 µl/disc). Origanum oil and thyme oil demonstrated moderate antifungal activities against conidial germination at similar levels. Both of them inhibited conidial germination in a dose-dependent manner. Increasing concentrations of origanum oil and thyme oil concentrations resulted in gradual decreases of conidial germination. No germination was found in conidia treated with 5 µl/disc of thyme oil.

**Different antifungal efficacies of four plant essential oils against *in vitro* mycelial growth of *F. oxysporum* f. sp. *fragariae*.** All four plant essential oils also possessed antifungal activities against mycelial growth of *F. oxysporum* f. sp. *fragariae* in a dose-dependent manner (Fig. 2). Cinnamon oil, origanum oil and thyme oil showed similarly elevated mycelial growth-inhibiting activities with increasing doses. Antifungal efficacy of cinnamon oil at 0.5 µl/disc was approximately 89.4% compared to untreated control. Increasing its dose to 1 µl/disc did not augment its antifungal activity. However, relatively higher doses of cinnamon oil at 2 µl/disc and 5 µl/disc resulted in significant reduction in mycelial growth (to approximately 60.0% and 6.9%, respectively). At least 0.2 µl/disc of origanum oil and thyme oil was needed to inhibit mycelial growth. Higher concentrations reduced mycelial growth more effectively. At the highest dose (5 µl/disc), origanum oil and thyme oil completely inhibited mycelial growth. By contrast, fennel oil had relatively lower antifungal activity with limited ability to arrest mycelial growth with increasing doses. Even at the highest dose (5 µl/disc), it only slightly reduced mycelial growth (approximately 84.4% compared to the untreated control).
Synergistic antifungal effects of plant essential oils in combination on *Fusarium oxysporum* f. sp. *fragariae* growth.

Different combinations of these four plant essential oils were applied to investigate whether they might have synergism in suppressing conidial germination and mycelial growth of *Fusarium oxysporum* f. sp. *fragariae* (Fig. 3, 4). Several combinations of these plant essential oils synergistically suppressed conidial germination (Fig. 3A). Treatment with cinnamon oil alone almost completely suppressed conidial germination. Additional treatment with other plant essential oils did not significantly increase the high suppressive activity of cinnamon oil. Three different combinations of two different essential oils (fennel oil + origanum oil, fennel oil + thyme oil, origanum oil + thyme oil) significantly increased their antifungal activities against conidial germination compared to any single plant essential oil treatment. No additional increase in antifungal activity against conidial germination was found by mixing three or four plant essential oils (Fig. 3B).

Enhanced suppressive activities against fungal mycelial growth were demonstrated by combined treatments with different plant essential oils (Fig. 4). After treatments with different combinations of essential oils, colony formation on PDA was demonstrated at 6 days after culture (Fig. 4A). Cinnamon oil treatment alone slightly reduced mycelial growth. Additional application with fennel oil failed to enhance its antifungal activity. However, addition application with origanum oil or thyme oil accelerated mycelial growth restriction caused by cinnamon oil. Single treatment with origanum oil or thyme oil drastically suppressed mycelial growth. Combined treatment with fennel oil and origanum oil showed synergistic effect on the suppression of mycelial growth compared to single treatment. Triple treatment with cinnamon oil, fennel oil and origanum oil resulted
in more reduction of mycelial growth. Antifungal activity against mycelial growth by triple treatment with cinnamon oil, origanum oil and thyme oil was similar to that by dual treatment with origanum oil and thyme oil. Triple treatment with cinnamon oil, fennel oil and thyme oil exerted additive antifungal activity compared to treatment with a combination of any two plant essential oils. Fennel oil treatment did not enhance the antifungal activity of dual treatment with origanum oil and thyme oil. Antifungal activity with a combination of all four essential oils was not significantly different from that with a combination of origanum and thyme oil (Fig. 4B).

**Discussion**

Due to their antifungal activities, plant essential oils have been applied in eco-friendly postharvest management to extend transportation and storage periods as well as shelf life of strawberry fruits in markets (Bhaskara Reddy et al., 1998; Nabigol and Morshed, 2011). Several fungal species such as *Botrytis cinerea*, *Rhizopus stolonifer* and *Aspergillus niger* frequently deteriorating strawberry fruit quality have been controlled by various plant essential oils. However, control method to mitigate Fusarium wilt occurrence in strawberry plant culture fields is limited in spite of growing concerns of its worldwide spread. Antifungal activities of several plant essential oils against *Fusarium oxysporum* causing Fusarium wilt in a variety of crop species have been evaluated. Clove oil has been found to be more effective in inhibiting *in vitro* conidial germination and mycelial growth of *F. oxysporum* f. sp. *lycopersici* causing tomato Fusarium wilt, compared to essential oils from lemongrass, mint, or eucalyptus plants (Sharma et al., 2017). Tomato Fusarium wilt disease has been efficiently reduced by treating soil mixtures with aqueous emulsion of clove oil (La Torre et al., 2016; Sharma et al., 2017). In this study, different antifungal activities of four plant essential oils against strawberry fungus *Fusarium oxysporum* f. sp. *fragariae* were demonstrated *in vitro*. Our results suggest that these essential oils might be useful to reduce Fusarium wilt in strawberry growing fields. These four plant essential oils (cinnamon oil, fennel oil, origanum oil and thyme oil) tested in this study showed different antifungal efficacies against *in vitro* conidial germination and mycelial growth of *F. oxysporum* f. sp. *fragariae*. Cinnamon oil was found to be the most effective one in suppressing conidial germination of *F. oxysporum* f. sp. *fragariae* among these four plant essential oils. Origanum oil and thyme oil were more effective in reducing mycelial growth compared to the other two essential oils. These results suggest that various plant essential oils have different antifungal activities against conidial germination and mycelial growth. Cinnamon oil has been widely used to inhibit the growth of various phytopathogenic fungi. It has been reported that conidial germination and mycelial growth of *F. oxysporum* f. sp. *cubense* can be significantly suppressed by cinnamon oil, resulting in decreased panama disease in banana seedlings (Monteiro et al., 2013). Therefore, cinnamon oil treatment might be useful to halt early development of Fusarium wilt in strawberry fields via inhibiting conidial germination in soil environments.
Origanum oil and thyme oil have not been applied to control *F. oxysporum* fungus yet. Both origanum oil and thyme oil can reduce spore germination and mycelial growth of postharvest destructive fungus *Monilinia fructicola* causing brown rot in stone fruits, including peaches and plums (Lazar-Baker et al., 2011). It has been reported that volatile origanum oil treatment can inhibit conidial germination, germ tube elongation and mycelial growth of grey mould fungus *Botrytis cinerea* (Soylu et al., 2010). Grey mould occurrence in tomato plants grown under greenhouse conditions can also be reduced via curative and protective effects of origanum oil (Soylu et al., 2010). Thyme oil can reduce in vitro growth of *Phytophthora infestans* and late blight of tomato plants in growing fields (El-Mohamedy and Abd-El-latif, 2015). Dose-dependent mycelial inhibition of *F. oxysporum f. sp. fragariae* by origanum oil and thyme oil found in this study suggest that they might be useful to manage Fusarium wilt of strawberry plants.

Commercial products containing different plant essential oils as a component have been available for crop protection. It has been reported that combined treatment with rosemary oil, clove oil and thyme oil can reduce tomato Fusarium wilt much more efficiently in comparison with control effect with single treatment by clove oil, thyme oil, or rosemary oil (La Torre et al., 2016). This provides evidence that different plant essential oils can be used simultaneously to enhance their antifungal activity and crop protection efficacies. In the present study, combined treatment with origanum oil and thyme oil (1:1, vol/vol) resulted in the best synergistic effect against mycelial growth of *F. oxysporum f. sp. fragariae* than other combinations. However, antifungal activities of origanum oil and thyme oil mixture at different ratios need to be elucidated to increase their antifungal efficacy.

To apply plant essential oils into crop fields practically, side effects including phytotoxicity should be considered. It has been shown that excess cinnamon oil can result in severe tissue damage, leading to detached tomato leaves (Lee et al., 2012). Dose-dependent phytotoxicities of soil-drenching clove oil to cucumber, muskmelon, pepper and tomato seedlings before transplanting have also been reported (Meyer et al., 2008). Therefore, phytotoxic effect of plant essential oils should be defined before applying them in strawberry fields regarding their dosage, frequency and application method as well as plant cultivar, age and organs.

In summary, our results revealed that the four plant essential oils tested in this study could efficiently suppress conidial germination and mycelial growth of *F. oxysporum f. sp. fragariae in vitro*. These effects were more evident when two plant essential oils were mixed together compared to single plant essential oil treatment. Therefore, developing commercial products containing several plant essential oils might be promising as eco-friendly strategy to control strawberry Fusarium wilt.

**Acknowledgments**

This research was supported by Gyeongnam National University of Science and Technology (GNTech) Grant 2016 to J. K. H.

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